Effects of projected near-future carbon dioxide levels on cephalopod physiology and behaviour

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This thesis is dedicated to my father, Dennis Neal Spady.

Declaration of Ethics

The research presented in this thesis was conducted in accordance with the JCU Animal Ethics Committee (A2189). Collection permits for study species were acquired from the Queensland Department of Agriculture, Fisheries and Forestry (170251).

Statement on the Contribution of Others

This thesis includes collaborative work with my advisors Dr. Sue-Ann Watson and Prof. Philip Munday. While undertaking these collaborations I was responsible for experimental design, animal collection and care, data collection, analysis, and interpretation of my results. My co-authors provided intellectual guidance, editorial assistance, statistical assistance, financial support, and technical assistance. **Chapter 2** includes collaborations with Dr. Jodie Rummer and Tiffany Nay who contributed in experimental design and editorial assistance.

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General Abstract

Oceanic uptake of anthropogenic carbon dioxide (CO_2) is causing changes to seawater chemistry, a process known as ocean acidification. This has the potential to negatively affect a wide variety of marine organisms. To date, most studies into the effects of elevated CO_2 on marine organisms have focused on highly calcified invertebrates and fishes, with a variety of effects observed, including impacts on early life history development, respiration, behaviour, and learning. Despite their importance in marine ecosystems, comparatively little is known about the possible effects of rising CO_2 levels on cephalopods. In this thesis, I investigate the effects of CO_2 levels projected for the end of this century on the aerobic scope, reproduction, development, predatory behaviours, and learning in two species of tropical cephalopod from different taxonomic orders, the two-toned pygmy squid (*Idiosepius pygmaeus*) and the bigfin reef squid (*Sepioteuthis lessoniana*).

In **Chapter 2**, I investigate the effects of elevated CO_2 on the minimum and maximum oxygen uptake rates, and aerobic scope of both *I. pygmaeus* and *S. lessoniana*. Due to the pH sensitive oxygen transport of cephalopods, I expected adverse effects on oxygen uptake rates. However, there were no effects of elevated CO_2 exposure on oxygen uptake rates or aerobic scope. This suggests a level of tolerance to elevated CO_2 in both species. The ability of these two cephalopod species to cope with long-term exposure to elevated CO_2 without detriment to their aerobic scope suggests that the energy available to partition to other important tasks, such as reproduction and growth, may remain optimal at elevated CO_2.

One of the more commonly observed effects of elevated CO_2 on marine molluscs is altered reproduction and early life history development. In **Chapter 3,** I test the effects of elevated CO_2 on reproduction and the potential for effects after parental exposure on early development in *I. pygmaeus*. Despite the lack of effects of elevated CO_2 on the aerobic scope of adults observed in **Chapter 2**, there were adverse effects of elevated CO_2 on reproduction and development. Parents held at elevated CO_2 produced clutches with 40% fewer eggs compared with parents in ambient control conditions. Embryo area, measured directly after spawning, was significantly reduced in the elevated CO_2 treatment compared to controls, and remained smaller throughout development. There was no effect of elevated CO_2 on fertility, time to hatch, or hatching success. Lastly, female squid laid eggs >40% closer together at elevated CO_2 compared with the controls, indicating a possible effect of elevated CO_2 on egg-

laying behaviour. The negative effects observed here appear to be predominately a response to parental exposure to elevated CO_2 rather than exposure of the eggs themselves. As the potential for adaptation is strongly influenced by reproductive success, these effects could have consequences to the capacity of *I. pygmaeus* to cope with future ocean conditions.

In **Chapter 4,** I investigate the effects of elevated CO_2 on the predatory behaviours of both *I. pygmaeus* and *S. lessoniana*. Both species exhibited an increased latency to attack prey and displayed altered body pattern choice during the attack sequence at elevated CO_2. *I. pygmaeus* also exhibited a 20% decrease in predation rate, an increased striking distance, and reduced preference for attacking the posterior end of the prey at elevated CO_2. The effects of elevated CO_2 on predatory behaviours and strategies seen here could have far-reaching consequences in marine ecosystems due to the important role of squid in marine food webs as both predator and prey.

Cephalopods have some of the most advanced learning capabilities among invertebrates. In **Chapter 5**, I investigate the effects of elevated CO_2 on the learning and capacity for conditional discrimination in *S. lessoniana*. Here, individuals were subjected to a three-task training and learning experiment within a two-choice maze containing visual cues. Performances throughout these three tasks were compared to determine the effects of elevated CO_2 on learning and conditional discrimination. Fifty percent of squid from both CO_2 treatments demonstrated conditional discrimination, and time to exit the maze as well as rate of correct choice was unaffected by elevated CO_2. These findings demonstrate conditional discrimination for the first time in a squid and suggest that the learning and memory capabilities of *S. lessoniana* will not be affected by future elevated CO_2 conditions.

This thesis demonstrates for the first time in any cephalopod species, the effects of elevated CO_2 on aerobic recovery, reproduction, predatory behaviours, and learning. The results show that while tolerant in some aspects of physiology and behaviour, other important traits are adversely affected by elevated CO_2. However, cephalopods have short lifespans, fast growth rates, large populations, and a high intrinsic rate of population increase - all traits that favour potential adaptation to changes in their natural environment. Future studies should investigate the capacity for these animals to adapt to elevated CO_2 and determine their potential to cope with future changes in ocean conditions over the timescale at which they are predicted to occur.

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Chapter 1: General Introduction

1.1 – Cephalopods: natural history, diversity, and biological adaptations

Cephalopods have been a subject of human fascination for several thousands of years. Ancient Greek pottery from circa 1500 BC had octopuses as a common theme and there is even an Egyptian hieroglyph of a squid (Neer, 2012). The first person to study biology systematically, Aristotle, had a particular interest in cephalopods and some of his descriptions and observations pertaining to them (e.g., the hectocotylus of octopuses) were widely disbelieved until the 19th century (Leroi, 2014). Throughout recorded history, cephalopods have stimulated scientific minds, inspired artistic emotions, and satisfied culinary senses. In fact, they even played a part in the act of recording history itself, as cuttlefish ink was a popular medium for writing, drawing, and painting in Greco-Roman times (Derby, 2014). As we learn more about them, the fascination from the general population seems only to increase. Perhaps this is because few living taxa, if any, are quite as unique as the cephalopod.

The earliest ancestors of modern cephalopods appear in the fossil record about 530 mya and a great diversity in species emerged roughly 500 mya (Kröger *et al*., 2011). Over the next 360 million years, cephalopods dominated the oceans as the top predators and diversified into over 17,000 species (Bandel *et al*., 1983). However, by the end of the Cretaceous period (~66 mya) teleost fishes began to challenge the long reign of cephalopods by competing for resources and predating upon them. Ancient cephalopods had heavy external shells and were likely poorly matched competitors for the new, swift swimmers. As the two taxa had overlapping niches, a coevolutionary arms race had begun (Packard, 1972). Eventually, the

fishes came to dominate the ocean, and most cephalopods evolved to be quite different from their earliest ancestors. Today, there are about 800 extant species of cephalopod of which only eight retained external shells. The remaining species are soft bodied animals, making them extremely vulnerable to predation. This may seemingly propose an inevitable demise, until one takes note of the many incredible and unique adaptations of cephalopods, which are a direct result of these competitive adaptations.

Coleoid cephalopods, comprising of squids, cuttlefishes, and octopuses, (hereafter referred to as only 'cephalopods') evolved such a range of unique and effective adaptations that, despite their wide variety of predators and lack of external protection, they remain an extremely successful class of animals today. One of the most notable adaptations is the strategy of "life in the fast lane", or living fast and dying young (Boyle & Rodhouse, 2005). Contributing to this is short lifespans (from 80 days to a few years), rapid growth rates, large populations, and a high intrinsic rate of population increase. This is arguably one of their most important traits, as it is what allows them to be such an abundant, widespread, and adaptable class of animals (Doubleday *et al*., 2016).

One impressive example of cephalopod evolution is their advanced vertebrate-like eyes. This influences their behaviour, contributing to their predatory and predator avoidance skills, and benefits their rapid spatial and social learning capabilities (Hanlon & Messenger, 2018). Further examples of unique adaptations within cephalopods are plentiful. The cells and organs used for camouflage (chromatophores, iridophores, leucophores, and papillae) enable rapid changes to the colour, shade, pattern, and even the texture of their skin, making cephalopods some of the most effective cryptic animals in the world. These are also used to

threaten and confuse predators, to conceal themselves from or to confuse prey, and for social interactions with conspecifics (Moynihan & Rodaniche, 1982; Hanlon & Messenger, 2018). Also to avoid being preyed upon, cephalopods can eject ink to mask themselves from or to confuse predators (Caldwell, 2005; Hanlon & Messenger, 2018). Inking can additionally be used to avoid detection by prey and as a conspecific alarm cue (Wood *et al*., 2008; Sato *et al*., 2016). While cephalopods utilise several modes of locomotion, their most unique is possibly jet propulsion, achieved by the constriction of mantle muscles forcing water through a small directable siphon. This allows them to rapidly change position in almost any direction after minimal notice. Lastly, is the large and complex brain of cephalopods. The brain to body size ratio in cephalopods is comparable to that of many vertebrates, including birds, and is greatest among almost all invertebrate taxa (Mather & Kuba, 2013). This allows for the processing of large amounts of information from their complex sensory systems as well as high-level learning and memory capabilities (Hanlon & Messenger, 2018). These adaptations, along with many others, give cephalopods much needed advantages over their vertebrate competitors. However, while cephalopods have benefited greatly from these traits, other traits such as some related to their aerobic capabilities may be at an evolutionary limit and could place cephalopods in a vulnerable position.

Cephalopods, and squid in particular, have high mass-specific oxygen consumption rates and their blood has low oxygen-carrying capacity compared to fishes (O'Dor & Webber, 1986; Shadwick *et al*., 1990). Their unique adaptation of jet propulsion, while very beneficial in predation, locomotion, and escape, is inherently inefficient and requires a large output of energy from their mantle muscles, further increasing their already high demand for oxygen (O'Dor, 1988a, 1988b). Another interesting evolutionary adaptation attempts to alleviate this

problem; squid have three hearts, two of which are purely for pumping large amounts of blood through their gills (Packard, 1972). However, both while active and at rest, some species are known to extract most or all of the oxygen from their blood on just one cycle through their body, leaving little to no venous oxygen reserve (O'Dor & Webber, 1986; Wells *et al*., 1988; Rosa & Seibel, 2008). These factors are said to force squid to chronically live at the edge of their environmental oxygen limitation (Pörtner, 2002). Furthermore, the oxygen carrying capacity of cephalopod hemocyanin (a protein found in molluscs that delivers oxygen to the tissues and cells) is highly sensitive to changes in pH (Brix *et al*., 1989, Bridges, 1995, Rosa & Seibel, 2008; Seibel, 2016). Unfortunately, the environment in which cephalopods live is currently at risk of a rapid decrease in pH due to increased anthropogenic carbon dioxide (CO_2) emissions. This human-induced change to the marine environment could therefore impact, among other things, the respiratory capabilities of cephalopods. That is to say, the future and survival of a class of animals that have inhabited the oceans for over half a billion years, and have fascinated humans for over 3,000 years, could be dramatically affected by anthropogenic activities in just a few centuries.

1.2 – Atmospheric CO_2 and ocean acidification

The Industrial Revolution completely revamped the lifestyles of humans worldwide and forever altered the course of civilization, but it did not come without consequences. In the roughly 250 years since the start of the Industrial Revolution, atmospheric CO_2 concentrations have increased by over 40%, from around 280 ppm (Joos & Spahni, 2008) to currently over 400 ppm (Dlugokencky & Tans, 2018; Le Quéré *et al*., 2018), and are now higher than any time in the previous 800,000 years (Lüthi *et al*., 2008). Furthermore, atmospheric CO_2 concentrations are increasing at least an order of magnitude faster than at any time in the

past million years (Doney & Schimel, 2007). If the current emissions trajectory continues under representative concentration pathway (RCP) 8.5, it is projected that CO_2 concentrations in the atmosphere could exceed 900 ppm by the end of this century (**Fig 1.1**), causing a reduction in ocean pH by a further 0.3-0.4 units (Collins *et al*., 2013). However, with governmental policy changes coming into effect and increasing global concern, less severe RCPs (4.5 or 6.0) for the end of the century may become more likely (Collins *et al*., 2013). Nearly one third of the anthropogenic CO_2 released into the atmosphere is absorbed by the oceans (Sabine & Feely, 2007), and as the partial pressure of CO_2 (pCO_2) in surface oceans are in approximate gas equilibrium with atmospheric CO_2 , they continue to rise at the same rate (Doney, 2010). Furthermore, when CO_2 combines with seawater, the net effect is an increase in CO_2, hydrogen ions, and bicarbonate ions, and a decrease in carbonate ions (Hoegh-Guldberg *et al*., 2007); this in effect causes a decrease in oceanic pH, which led to the term, 'ocean acidification'.

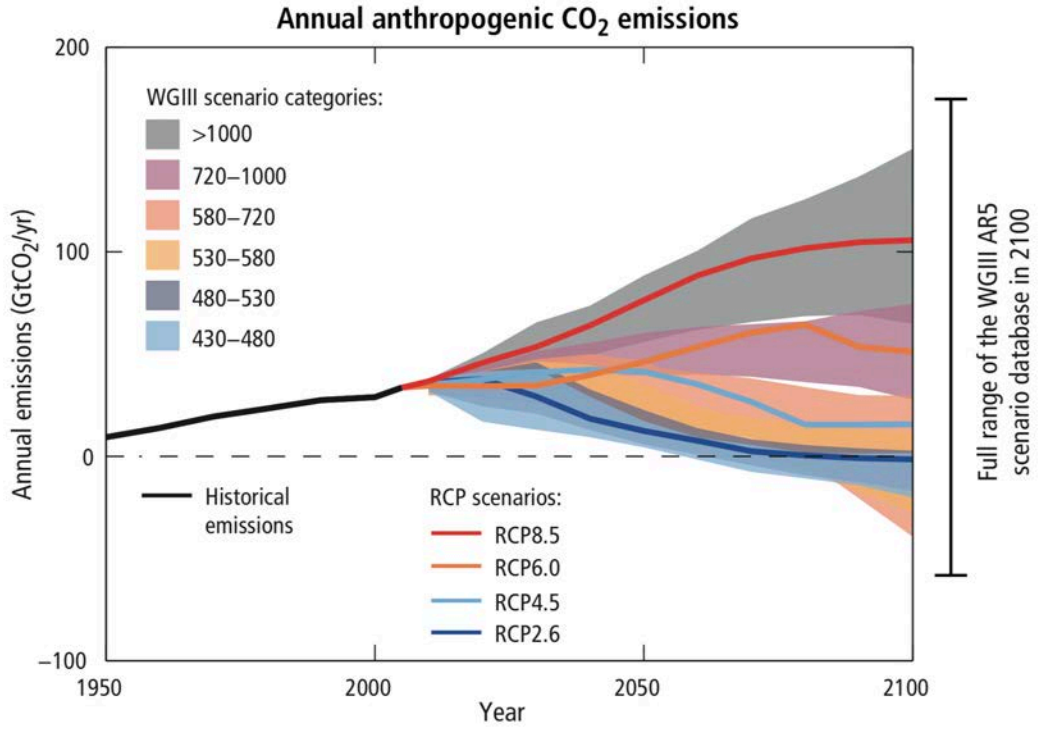

Fig 1.1 – Carbon dioxide emissions and associated RCPs with the range of emission scenarios (from IPCC, 2014).

1.3 – Effects of elevated CO_2 on marine animals

Physiological effects

The rapid changes to ocean chemistry predicted from rising CO_2 levels has the potential to adversely affect the physiology of a wide range of marine species and have considerable impacts on marine ecosystems (Hoegh-Guldberg *et al*., 2007; Sabine & Feely, 2007; Fabry *et al*., 2008; Pörtner & Farrell, 2008; Doney *et al*., 2009). One of the major concerns of climate change on active marine species is the costs of elevated CO_2 on oxygen uptake, and more broadly, aerobic performance. It has been hypothesised that the capacity to deliver sufficient oxygen to the tissues, in order to meet increasing demand, can constrain the performance of marine species under climate change (Pörtner & Farrell, 2008). A metric commonly used as an indicator of an individual's performance is aerobic scope, defined as the difference between the routine oxygen uptake (routine $\dot{M}O_2$) and maximal oxygen uptake ($\dot{M}O_{2Max}$) rates. An animal's aerobic scope is indicative of the amount of oxygen available, beyond basal metabolic costs, that can be allocated to critical aerobic activities, such as growth, reproduction, foraging, and locomotion (Eliason *et al*., 2008; Pörtner & Farrell, 2008; Pörtner & Peck, 2010).

The type of effect of elevated CO_2 on aerobic scope, as well as its magnitude, appears to be species specific. For example, some fish species respond to elevated CO_2 with a decrease in aerobic scope (Munday *et al*., 2009a), while others are not affected (Melzner *et al*., 2009a), and some species even respond with an increased aerobic scope (Couturier *et al*., 2013; Rummer *et al*., 2013). Recent meta-analyses, considering all studies conducted to date, have concluded that there is no consistent effect of elevated CO_2 on aerobic scope in fishes (Lefevre, 2016; Cattano *et al*., 2018; Hannan & Rummer, 2018). In molluscs, few studies have

investigated the effects of elevated CO_2 on aerobic scope, however, within these studies the type of effects also vary. The scallop, *Pecten maximus*, responds to elevated CO_2 with a reduced aerobic scope (Schalkhausser *et al*., 2013), while the gastropod, *Gibberulus gibberulus gibbosus*, is unaffected (Lefevre *et al*., 2015).

In cephalopods, although many studies have investigated the effects of elevated CO_2 on aerobic performance, no studies have investigated the effects of elevated CO_2 on aerobic recovery, and only one study has determined aerobic scope (Rosa & Seibel, 2008). Nevertheless, the effects of elevated CO_2 appear to again be species specific, and also dependent on life-stage within some species. Routine oxygen uptake ($\dot{M}O_2$) of juvenile jumbo squid, *Dosidicus gigas*, is suppressed by ~20% under elevated CO_2 (Rosa & Seibel, 2008), whereas routine $\dot{M}O_2$ in juveniles of the common cuttlefish, *Sepia officinalis*, is unaffected at extreme CO_2 levels (~6,000 µatm) (Gutowska *et al*., 2008). However, while juvenile *S. officinalis* show metabolic tolerance to elevated CO_2, individuals in the earlier life history stages (late stage incubation period) demonstrate reduced oxygen uptake by ~20% at elevated CO_2 (Rosa *et al*., 2013; Sigwart *et al*., 2016). As adults, the bigfin reef squid, *Sepioteuthis lessoniana*, are not affected by CO_2 levels of 1586 µatm, but oxygen uptake rates are depressed by 40% at 4134 µatm (Hu *et al*., 2014). The range of effects on routine $\dot{M}O_2$ among species and life stages makes it difficult to predict the effects on aerobic scope. However, if aerobic scope is adversely affected, it could constrain the amount of oxygen available for other important tasks, such as reproduction and growth.

Tolerance to elevated CO_2 is highly dependent on an animal's life stage (Melzner *et al*., 2009b), and this appears to hold true for some cephalopod species with respect to their

oxygen uptake capabilities. In general, stress from elevated CO_2 is expected to be particularly severe during the early development and reproductive stages of marine animals, while adults are expected to be more tolerant (Ishimatsu *et al*., 2004; Melzner *et al*., 2009b; Kroeker *et al*., 2010). In a meta-analysis by Kroeker *et al*. (2013), 228 studies on the physiological effects of elevated CO_2 on a range of marine taxa revealed that effects on early life history traits were among the most common. Furthermore, molluscs were among the most sensitive taxa with the widest range of adverse responses to elevated CO_2 (Kroeker *et al*., 2013). In a similar meta-analysis focusing specifically on molluscs, 16 of 17 gastropod and bivalve species responded negatively to elevated CO_2 in relation to their embryonic and larval development (Parker *et al*., 2013). Not only are animals usually more sensitive during development and reproductive periods, but physiological stress from elevated CO_2 during the early ontogeny of animals has been described as the "true bottleneck" through which species must pass in order to successfully cope with future elevated CO_2, even when the species demonstrates tolerance in other traits (Melzner *et al*., 2009b). However, most of the molluscs investigated to date are highly calcifying species (e.g., gastropods and bivalves) and the effects of elevated CO_2 on the developmental traits of cephalopods, while not absent (e.g. Rosa *et al*., 2014; Navarro *et al*., 2014; 2016), have not been as thoroughly investigated. Moreover, effects of elevated CO_2 on cephalopod reproduction has not been investigated at all.

Behavioural effects

While the primary concern about the effects of elevated CO_2 on marine organisms has been on physiological responses, studies have also started to investigate the effects on animal behaviour. Recent studies have shown that elevated CO_2 affects a variety of behaviours in a wide range of marine species (Nagelkerken & Munday, 2016). Most of the work on elevated

CO_2 and behaviour has focused on reef fishes (Heuer & Grosell, 2014), showing multiple adverse effects to sensory systems (Munday *et al*., 2009b; Munday *et al*., 2010; Simpson *et al*., 2011) and alterations to ecologically important behaviours, such as predator-prey interactions (Cripps *et al*., 2011; Ferrari, 2011a; Allan *et al*., 2013; Ferrari *et al*., 2015; Pistevos *et al*., 2015; Pistevos *et al*., 2016). Although less studied, similar effects such as altered activity, predator avoidance, and predatory behaviours have been demonstrated in gastropod molluscs (Bibby *et al*., 2007; Manríquez *et al*., 2013; Manríquez *et al*., 2014; Watson *et al*., 2014; Jellison *et al*., 2016; Watson *et al*., 2017). Apart from the studies within this thesis, only two have investigated the effects of elevated CO_2 on cephalopod behaviour (Spady *et al*., 2014; Zakroff *et al*., 2017). In the two-toned pygmy squid (*Idiosepius pygmaeus*), elevated CO_2 altered defensive responses to a visual predator cue. Squid at elevated CO_2 were more likely to ink and perform a jet escape, as well as to display altered body pattern choice (Spady *et al*., 2014). However, the time to react to the visual cue and the distance moved were not affected by elevated CO_2, indicating that the physiological ability to escape was not affected. Instead, altered predator escape responses were likely due to an effect of elevated CO_2 on decision making processes. As most squid occupy a very important position in marine ecosystems, as both predator and prey, it would be useful to determine if elevated CO_2 has an effect on their predatory behaviours as well.

Another ecologically relevant behaviour that may be at risk from elevated CO_2 is learning and memory. Elevated CO_2 has been shown to inhibit learning in the tropical damselfish, *Pomacentrus amboinensis*, causing them to be unable to learn to appropriately respond to a common predator fish (Ferrari *et al*., 2012). Learning and memory in the temperate fish, *Gasterosteus aculeatus*, is also adversely affected by elevated CO_2, with reduced arena escape

times over successive trials (Jutfelt *et al*., 2013). In *P. amboinensis*, inhibited learning resulting from elevated CO_2 has been linked to the $GABA_A$ receptor, a major inhibitory neurotransmitter receptor (Chivers *et al*., 2014). $GABA_A$ receptors, along with other inhibitory mechanisms, are important for learning processes in squid (Conti *et al*., 2013). For example, $GABA_A$ is likely to be involved in the experience-dependent learning of prey capture and escape responses, which is highly dependent on the inhibitory control of the giant synapse output (Preuss & Gilly, 2000). Although the effect of elevated CO_2 on learning has not been investigated in any invertebrate species, the effects on predator escape responses in the gastropod mollusc, *Gibberulus gibberulus gibbosus*, has been linked to interference with $GABA_A$-like receptors (Watson *et al*., 2014). The previously mentioned behavioural effects of elevated CO_2 on *I. pygmaeus* share similarities to the behavioural changes linked to $GABA_A$ in gastropods. This indicates some potential for a similar mechanism responsible for the change in behaviour. If these effects on squid behaviour at elevated CO_2 are due to interference with $GABA_A$ function, effects on squid learning and memory may be possible as well.

Cephalopods occupy a central position in many marine food webs. They prey upon just about anything that they can capture (teleost fishes, crustaceans, bivalves, echinoderms, other cephalopods, and small planktonic species), and a wide variety of marine taxa view them as prey, including cetaceans, pinnipeds, marine birds, teleost fishes, sharks, and turtles (Hanlon & Messenger, 2018). Considering this, it is safe to assume that changes to the physiological performance, reproductive capacity, predator-prey interactions, or learning of cephalopods could have drastic implications for ecosystem structure. Determining the effects of elevated CO_2 on cephalopod physiology and behaviours is helpful in understanding and predicting the effects of ocean acidification on marine ecosystems.

1.4 – Thesis outline and general methods

Study species

This thesis examines the potential for elevated CO_2 to affect the metabolic and reproductive physiology and behavioural ecology of two species of tropical cephalopod from separate taxonomic orders. The two species utilised throughout this thesis are the two-toned pygmy squid, *Idiosepius pygmaeus* (**Fig. 1.2**), and the bigfin reef squid, *Sepioteuthis lessoniana* (**Fig. 1.3**). One of the smallest and shortest lived cephalopod species, *I. pygmaeus* (**Chapters 2**, **3**, and **4**) has a mantle length of <20 mm and a life span of about <90 days (Moynihan, 1983; Jackson, 1988). This species has a unique adhesive gland on the mantle, allowing it to attach to seagrass or flotsam where it can rest for extended periods (von Byern & Klepal, 2006). *I. pygmaeus* inhabits shallow coastal waters from northern Australia to the South China Sea, feeding mainly on small crustaceans, and may be an important food source for juvenile fishes (Jackson, 1991). A much larger and more active species, *S. lessoniana* (**Chapters 2**, **4**, and **5**) attains maximum mantle length sizes of up to 36 cm and occupies a wider range of habitats from open oceans to inshore areas and coral reefs (Norman, 2003). *S. lessoniana* also has one of the largest distributions of any inshore squid species (Jereb & Roper, 2005) and has a much wider range of both predators and prey when compared to *I. pygmaeus*. Both species have previously been the subject of elevated CO_2 experiments, with *I. pygmaeus* responding with altered activity and predator avoidance behaviours (Spady *et al*., 2014) and *S. lessoniana* used as a model for acid-base regulation in squids, as well as in investigations into the effects on routine oxygen uptake rates (Hu *et al*., 2013; Hu *et al*., 2014). Both species are also well suited for laboratory studies given the relative ease in caring for them in captivity compared with many other cephalopod species. Furthermore, despite being commonly called ‘pygmy squid’, *I. pygmaeus* is not a true squid species and is in the order Idiosepiida, whereas *S. lessoniana*

is in the order Teuthida making it a true squid. The taxonomic separation between the species is important in this thesis, as comparing effects between different orders can help inform the potential scope of elevated CO_2 effects in cephalopods more generally. Both species are distinct in their ecological role, physiology, and range of behaviours, making the use of the two separate species valuable in understanding a wider range of responses to elevated CO_2 conditions among species.

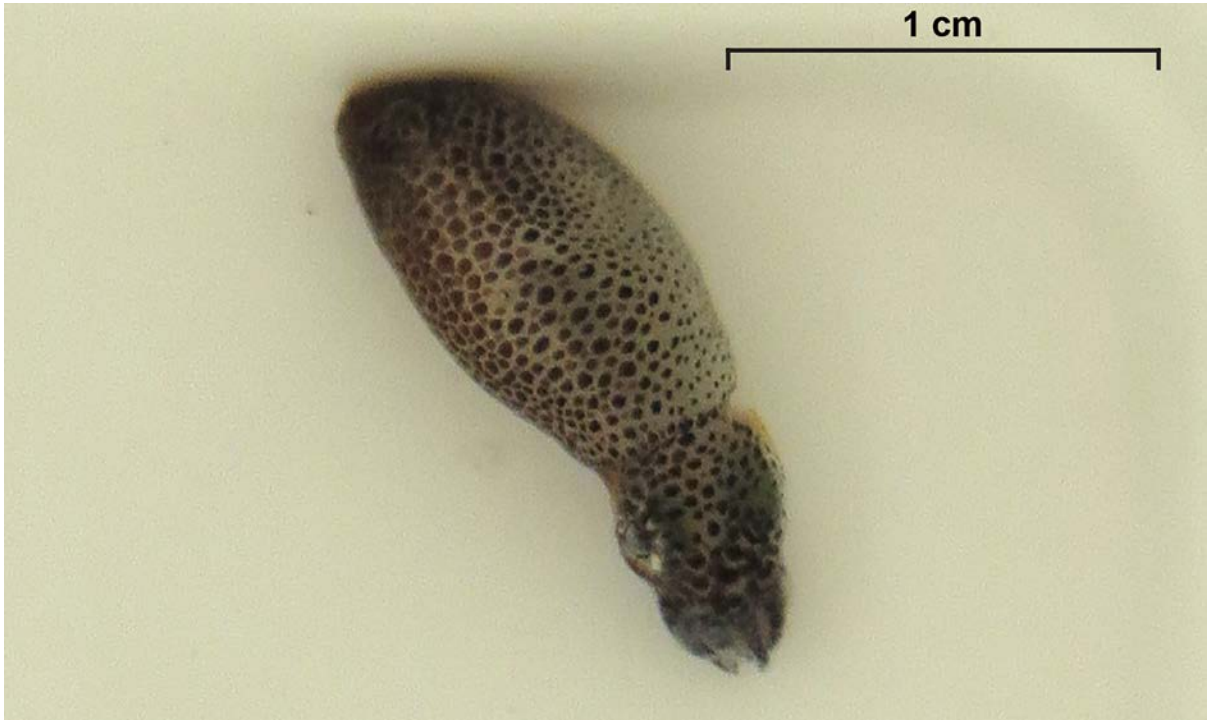

Figure 1.2 – The two-toned pygmy squid, *Idiosepius pygmaeus*.

Both species were captured from the Townsville breakwater (19˚24'S, 146˚82'E) by handheld dip-net. *I. pygmaeus* were captured year-round off of the western wall of the breakwater where there is abundant seagrass, the preferred habitat of *I. pygmaeus*. This species was also collected from the nearby Breakwater Marina. All individuals were captured in the morning between 0600 and 1000. *S. lessoniana* were captured each year between April and July, when

adults are more abundant, from both the western and eastern wall of the breakwater as well as at the mouth of the Ross Creek. All individuals were captured at night between 1900 and 0200.

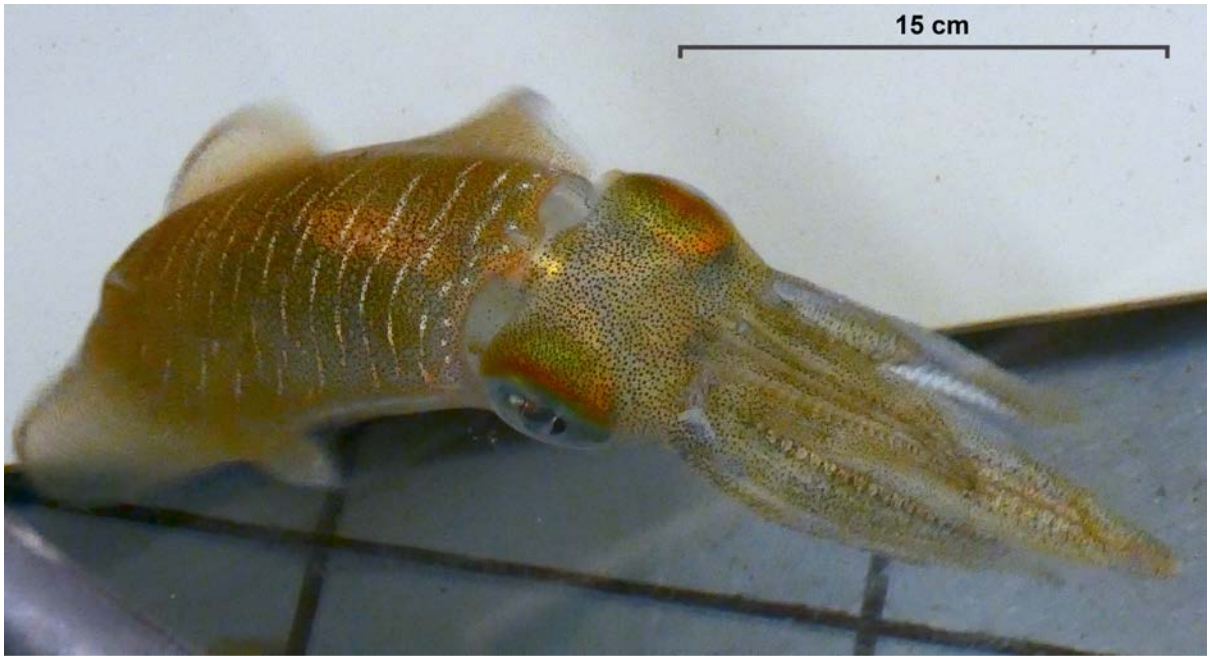

Figure 1.3 – The bigfin reef squid, *Sepioteuthis lessoniana*.

Aims

The purpose of this thesis is to determine the potential for effects of CO_2 levels projected for the end of this century under a worst case scenario (approximately 1,000 µatm) (Collins *et al*., 2013) on the physiology and behaviours of tropical cephalopods. To investigate this aim, two experiments address questions regarding the physiology of cephalopods after exposure to elevated CO_2, and two experiments address behavioural issues. In **Chapter 2**, the effects of elevated CO_2 on the aerobic performance of both *I. pygmaeus* and *S. lessoniana* are investigated. Specifically, the routine oxygen uptake rates, maximal oxygen uptake rates, aerobic scope, and recovery time of both species are measured and compared between

current-day control and elevated CO_2 treatments. The results from this chapter shed light on the effects of elevated CO_2 on cephalopod aerobic scope, which may help to explain effects on other performance traits. **Chapter 3** investigates the effects of elevated CO_2 on the reproduction of *I. pygmaeus* and development after parental exposure. Here, squid were exposed to either control or elevated CO_2 for 14 days prior to being paired and allowed to mate. Measurements of reproductive performance include: number of eggs per clutch, proportion of fertile eggs per clutch, distance between eggs within clutch, embryo size at three key developmental stages, proportion of eggs to hatch, time to hatch, and hatching period. This chapter expands on the known effects of elevated CO_2 on cephalopod development by taking into account the effects of parental exposure and measuring effects on reproduction.

The effects of elevated CO_2 on the predatory behaviours of both *I. pygmaeus* and *S. lessoniana* are investigated in **Chapter 4**. Here, after exposure to either control or elevated CO_2 treatments, squid interactions with a common prey species are observed in order to determine if elevated CO_2 has an effect on: likelihood to attack, latency to attack, time holding attack pose, striking distance, striking direction, body pattern choice, and first strike capture success. The effect of elevated CO_2 on activity was also investigated in *S. lessoniana* as a previous study demonstrated an effect of elevated CO_2 on activity in *I. pygmaeus*. Lastly, in **Chapter 5**, the effects of elevated CO_2 on the learning and capacity for conditional discrimination is investigated in *S. lessoniana*. Individuals are trained to connect certain visual cues with the correct exit in a two-choice maze. Then, visual cues are shown at random to see if individuals can discriminate between cues and choose the correct exit. Individuals from each CO_2 treatment are compared to see if elevated CO_2 had an effect on their ability to

choose the correct exit, indicating conditional discrimination, and if there are differences in their general performance throughout the experiment.

Together, these four data chapters advance our knowledge of the impacts of projected future CO_2 levels on cephalopods and the marine ecosystems that they inhabit. For the first time, the effects of elevated CO_2 are investigated on cephalopod aerobic recovery, reproduction, predatory behaviours, and learning. Examining the responses of these species to elevated CO_2 in the contexts investigated here improves our understanding of how marine communities as a whole might interact and respond to a rapidly changing environment.

Chapter 2: Aerobic performance of two tropical cephalopod species unaltered by prolonged exposure to projected future carbon dioxide levels

Blake L. Spady, Tiffany J. Nay, Jodie L. Rummer, Philip L. Munday, Sue-Ann Watson

This chapter is in review at *Conservation Physiology*.

2.1 – Summary

Squid and many other cephalopods live continuously on the threshold of their environmental oxygen limitations. If the abilities of squid to effectively take up oxygen are negatively affected by projected future carbon dioxide (CO_2) levels in ways similar to those demonstrated in some fish and crustaceans, it could affect the success of squid in future oceans. While acute exposure to elevated CO_2 can have adverse effects on cephalopod respiratory performance, no studies have investigated this in an adult cephalopod after relatively prolonged exposure to elevated CO_2. Here, I tested the effects of prolonged exposure (≥ 20% of lifespan) to elevated CO_2 levels (~1,000 µatm) on the routine and maximal oxygen uptake rates, aerobic scope, and recovery time of two tropical cephalopod species, the two-toned pygmy squid, *Idiosepius pygmaeus* and the bigfin reef squid, *Sepioteuthis lessoniana*. Neither species exhibited evidence of altered aerobic performance after exposure to elevated CO_2 when compared to individuals held at control conditions. The recovery time of *I. pygmaeus* under both control and elevated CO_2 conditions was less than one hour; whereas, *S. lessoniana* required approximately eight hours to recover fully following maximal aerobic performance. This difference in recovery time may be due to the more sedentary behaviours of *I. pygmaeus*. The ability of these two cephalopod species to cope with long-

term exposure to elevated CO_2 without detriment to their aerobic performance suggests they may be resilient to an increasingly high CO_2 world.

2.2 – Introduction

Squid, the most active order of cephalopods, have high mass-specific oxygen consumption rates and blood with low oxygen-carrying capacity when compared to fishes (O'Dor and Webber, 1986; Shadwick *et al.*, 1990). One of their most advantageous evolutionary adaptations, jet propulsion, allows them to rapidly escape predators and compete for food with carnivorous fishes (Hanlon & Messenger, 2018). However, this mode of locomotion is inherently inefficient, requiring a significant output of energy from their mantle muscles, which further increases their already high demand for oxygen (O'Dor, 1988a, 1988b). To satisfy such a high demand of oxygen, both at rest and during sustained swimming, squid must pump large amounts of blood and extract most (or all) of the oxygen from it during one cycle through the body, leaving little to no venous oxygen reserve (O'Dor & Webber, 1986; Wells *et al.*, 1988; Rosa & Seibel, 2008). The combination of these factors contribute to many squid species living chronically at the threshold of their oxygen limitation (Pörtner, 2002). Furthermore, the oxygen carrying capacity of cephalopod hemocyanin, which delivers the oxygen to tissues and cells, has been hypothesised to be reduced due to changes in pH (Brix *et al*., 1989; Bridges, 1995; Rosa & Seibel, 2008; Seibel, 2016). Environmental changes, such as elevated carbon dioxide (CO_2), therefore have the potential to impact their respiratory capabilities. However, models by Birk *et al*. (2018) suggest that climate change relevant CO_2 levels may not alter squid blood-oxygen binding to a degree that will affect aerobic scope.

Atmospheric CO_2 concentrations have increased from 280 ppm before the industrial revolution to over 400 ppm in the current-day (Dlugokencky & Tans, 2016), a level that is higher than any time in the past 800,000 years (Lüthi *et al.*, 2008). On the current emissions trajectory, it is projected that atmospheric CO_2 will exceed 900 ppm by the end of this century (Collins *et al.*, 2013), increasing at a rate at least an order of magnitude faster than at any time in the past million years (Doney & Schimel, 2007). The partial pressure of CO_2 (pCO_2) in the surface oceans is in approximate gas equilibrium with that of the atmosphere, meaning as CO_2 concentrations in the atmosphere increase, concentrations in the oceans increase at approximately the same rate (Doney, 2010). Furthermore, along with diel fluctuations, the oceans experience seasonal fluctuations in pCO_2, and these fluctuations are projected to become amplified in the future due to the increased Revelle (buffer) factor of acidified seawater (McNeil & Sasse, 2016). The increase in average pCO_2 along with the amplification of seasonal cycles of pCO_2 indicates that marine organisms could experience CO_2 levels greater than 1,000 µatm before the end of the century (McNeil & Sasse, 2016). This rapid increase of CO_2 levels in the surface oceans could have a range of adverse effects on many marine species (Hoegh-Guldberg *et al.*, 2007; Fabry *et al.*, 2008; Doney *et al.*, 2009; Kroeker *et al.*, 2013; Clements & Hunt, 2015; Cattano *et al.*, 2018). Whether the oxygen uptake capabilities of squid will be susceptible to elevated CO_2 levels may depend on the level of sensitivity of their blood to changes in pH (e.g., Seibel, 2016; Birk *et al*., 2018).

The capacity to deliver sufficient oxygen to the tissues, in order to meet increasing demand, has been hypothesised to constrain the performance of marine species under climate change (Pörtner & Farrell, 2008). The physiological performance of an animal is often linked to their aerobic scope, the difference between the routine oxygen uptake (routine $\dot{M}O_2$) and maximal

oxygen uptake ($\dot{M}O_{2Max}$) rates. Aerobic scope indicates the amount of oxygen available, beyond basal metabolic costs, for critical aerobic activities (Eliason *et al.*, 2008; Pörtner & Farrell, 2008; Pörtner & Peck, 2010). The type of effect and magnitude of changes in aerobic scope resulting from elevated CO_2 appear to be species specific. For example, elevated CO_2 has been reported to decrease (Munday *et al.*, 2009a), increase (Couturier *et al.*, 2013; Rummer *et al.*, 2013), or have no effect on aerobic scope in fishes (Melzner *et al.*, 2009a). Moreover, recent meta-analyses find no consistent effect of elevated CO_2 on aerobic scope in marine fishes when all studies conducted to date are considered (Lefevre, 2016; Cattano *et al.*, 2018; Hannan & Rummer, 2018). In molluscs, there are similarly variable results, with elevated CO_2 reducing aerobic scope in the scallop, *Pecten maximus* (Schalkhausser *et al.*, 2013), yet no effect of elevated CO_2 on the aerobic scope of the gastropod, *Gibberulus gibberulus gibbosus* (Lefevre *et al.*, 2015). The effects of elevated CO_2 on mollusc routine $\dot{M}O_2$ also varies among species. Elevated CO_2 depresses oxygen uptake rates in six bivalve species (Fernández-Reiriz *et al.*, 2011; Wenguang & Maoxian, 2012; Navarro *et al.*, 2013), and one gastropod species (Melatunan *et al.*, 2011). However, other bivalve species respond to elevated CO_2 with an increase in oxygen uptake rates (Beniash *et al.*, 2010; Cummings *et al.*, 2011). Given the variety of effects in a range of mollusc taxa, most of which have a sedentary lifestyle, it is difficult to predict how the more active cephalopod species will respond to elevated CO_2.

In cephalopods, the effects of elevated CO_2 on respiratory performance appears to be both species as well as life stage specific. Very high CO_2 (~4,000 µatm) had no effect on routine $\dot{M}O_2$ in juvenile common cuttlefish, *Sepia officinalis* (Gutowska *et al.*, 2008). In the same species, similarly high CO_2 exposure (~3,600 µatm) reduced oxygen uptake by ~20% during

the late stage incubation period; although, there was no effect at CO_2 levels more closely resembling those that could occur in the ocean in the next 100 years (~1,400 µatm) (Sigwart *et al.*, 2016). In late stage embryos and newly hatched paralarvae of the squid, *Loligo vulgaris*, elevated CO_2 (~1,650 µatm) reduced oxygen uptake rates significantly (Rosa *et al*., 2014). The routine $\dot{M}O_2$ of adult bigfin reef squid (*Sepioteuthis lessoniana*) was not altered by moderately high CO_2 levels (1,586 µatm) (Hu *et al.*, 2014), but oxygen uptake rates were depressed by 40% when they were exposed to very high CO_2 levels (4,134 µatm) for seven days (Hu *et al.*, 2014). In juvenile jumbo squid, *Dosidicus gigas*, elevated CO_2 levels (estimated ~1,000 µatm) suppressed $\dot{M}O_{2Max}$ by ~30% and routine $\dot{M}O_2$ by ~20% (Rosa & Seibel, 2008). However, a recent study that used intermittent flow respirometry rather than flow-through respirometry found no effects of elevated CO_2 (1,410 µatm) on the same species (Birk *et al*., 2018). These results indicate a range of prospects for cephalopods, however, the effects of prolonged exposure of several weeks to climate change relevant CO_2 levels on the aerobic scope and recovery of cephalopods may provide a more ecologically relevant understanding.

In this study, I tested the effects of projected future CO_2 levels on the respiratory performance of two tropical cephalopod species. The two-toned pygmy squid (*Idiosepius pygmaeus*) has an average body length of <20 mm and inhabits shallow coastal waters from northern Australia to the South China Sea (Moynihan *et al*., 1983; Semmens *et al.*, 1995). This species has a unique adhesive gland on the mantle, allowing it to attach to seagrass or flotsam where it can rest for extended periods (von Byern & Klepal, 2006). These seagrass habitats in which *I. pygmaeus* can be found have the potential for diel fluctuations in CO_2 concentrations between ~80-700 µatm (Chou *et al*., 2018), indicating that the species may be able to tolerate periods of elevated CO_2 conditions. The second species, the bigfin reef squid, is a larger and

much more active species, found in tropical waters of up to 100 m, and are often found feeding in coral reef habitats at night (Norman, 2003). These two species, while both commonly called 'squid', are in fact from two separate taxonomic orders. The bigfin reef squid is a true squid of the order Teuthida; whereas, the pygmy squid is in the order Idiosepiida, which is more closely related to cuttlefish (Sepiida). The two species were subjected to a current-day control (396-440 µatm) or a projected future CO_2 level (997-1,039 µatm) for 18 (pygmy squid) or 75 (bigfin reef squid) days. Pygmy squid and bigfin reef squid live for approximately 90 and 210 days respectively, therefore, the treatment period was approximately 20% and 36% of the total lifespan of each species. I hypothesised that elevated CO_2 would reduce $\dot{M}O_{2Max}$ and aerobic scope in both cephalopod species, possibly due to interference to the oxygen carrying capacity of the blood as there is a high pH sensitivity of blood-oxygen binding in most active squids (e.g., Bridges, 1995). To determine this, I measured $\dot{M}O_{2Max}$ and routine $\dot{M}O_2$ via intermittent flow respirometry and then calculated aerobic scope ($\dot{M}O_{2Max}$ – routine $\dot{M}O_2$).

2.3 – Methods

CO_2 treatment systems

Experiments were conducted using 8,000 L recirculating seawater systems at James Cook University's research aquarium in Townsville, Australia. CO_2 levels were set at: 1) a current-day control (*Idiosepius pygmaeus*: 396 µatm; *Sepioteuthis lessoniana*: 440 µatm) and 2) an upper end-of-century projection following RCP8.5 (elevated CO_2, *I. pygmaeus*: 1,039 µatm; *S. lessoniana*: 997 µatm) (Collins *et al.*, 2013). A pH control system (AT Control, Aqua Medic, Germany) dosed CO_2 into 3,000 L sumps to achieve the desired pH level for each CO_2 treatment. pH on the NBS scale (pH_{NBS}) was measured daily (Seven2Go Pro, Mettler Toledo,

Switzerland) and dosing set points were adjusted as necessary to maintain the target pCO_2 in each treatment. Equilibrated seawater from each system was delivered at 1.5 L min^{-1} to tanks containing squid. Temperature was measured daily in each tank (Comark C26, Norfolk, UK).

Water samples were taken weekly to determine pH on the total scale (pH_T) by spectrophotometry (UVmini-1240, Shimadzu, Suzhou Instruments Co. Ltd., Kyoto, Japan) using m-cresol purple as an indicator dye (Dickson & Millero, 1987; Dickson *et al.*, 2007). Comparison of pH_{NBS} and pH_T in the weekly sample were used to estimate daily pH_T values. Total alkalinity was estimated weekly by Gran Titration (888 Titrando Metrohm AG, Switzerland) ensuring titration calibrations remained within one percent of certified reference material from Dr. A.G. Dickson (Scripps Institution of Oceanography, batch #135). Salinity was measured weekly using a conductivity sensor (HQ15d, Hach, Loveland, CO, USA). Carbonate chemistry parameters (**Table 2.1**) were calculated in CO2SYS (Pierrot *et al.*, 2006) using the constants K1, K2 from Mehrbach *et al*. (1973) refit by Dickson and Millero (1987) and Dickson *et al.* (2007) for $KHSO_4$.

Table 2.1 – Seawater carbonate chemistry – The temperature, salinity, $pH_{(T)}$, total alkalinity, and pCO_2 for each CO_2 treatment level and species. Total alkalinity and salinity values are from weekly measurements. Values are means ± SD.

Species	CO_2 Treatment	Temperature (˚C)	Salinity	$pH_{(T)}$	Total alkalinity (µmol/kg SW)	pCO_2 (µatm)
Idiosepius pygmaeus	Control	28.1 (±0.3)	36.7 (±1.1)	8.05 (±0.07)	2373 (±55)	396 (±76)
	Elevated	28.1 (±0.4)	36.4 (±0.9)	7.67 (±0.05)	2228 (±63)	1039 (±118)
Sepioteuthis lessoniana	Control	28.2 (±0.4)	36.3 (±0.4)	7.99 (±0.05)	2229 (±67)	440 (±60)
	Elevated	28.5 (±0.4)	36.0 (±0.7)	7.69 (±0.04)	2212 (±130)	997 (±110)

Experimental animals

Two-toned pygmy squid (wet mass, 0.25 ± 0.09 g; mantle length, 11.2 ± 1.7 mm; means ± SD) were collected by dip net (500 µm mesh) in March 2017 from Cleveland Bay in Townsville, Queensland, Australia (19˚24'S, 146˚82'E) and immediately transported to James Cook University, Townsville. Squid were maintained in round tanks (47Ø x 51H cm, 67 litres) at a maximum of five individuals per tank. Animals were provided with PVC pipe structures in the tanks as shelter. Animals were maintained at control conditions for 24-hours in holding tanks before being transferred to separate tanks of the same size that received a continuous flow of either control or elevated CO_2 treatment water. Glass shrimp (*Acetes sibogae australis*) were held at control conditions and provided *ad libitum* to squid every morning. Squid from both treatments were observed regularly feeding throughout the day, but food was withheld for 24h prior to experimentation to ensure a post-absorptive state that would maximise energy available for performance (Niimi & Beamish, 1974). Pygmy squid remained in either control (n=8) or elevated CO_2 (n=10) treatment for 18 days before respirometry trials. This treatment duration represents approximately 20% of the total pygmy squid lifespan.

Bigfin reef squid (wet mass, 181.2 ± 39.9 g; mantle length, 148.7 ± 13.7 mm; means ± SD) were collected in June 2016 from the Townsville breakwater, Queensland, Australia. Animals were captured at night with a 2.5 cm mesh, round frame dip-net and transported immediately to James Cook University, Townsville. Squid were kept individually in round tanks (47Ø x 51H cm) filled to 67-litres. Individuals were kept at control conditions for 21 days before they were transferred to separate tanks of the same size that received a continuous flow of either control or elevated CO_2 treatment water. Bigfin reef squid were fed a variety of live food, including locally caught estuary glassfish (*Ambassis marianus*), juvenile flathead grey mullet

(*Mugil cephalus*), as well as spiny chromis damselfish (*Acanthochromis polyacanthus*), twice daily. Feeder fish were maintained under control CO_2 conditions prior to being offered to the squid and were consumed equally by squid from both treatments. Bigfin reef squid remained in either control (n=8) or elevated CO_2 (n=7) treatment for 75 days before their respirometry trials. This treatment duration represents more than 35% of their average 208-day lifespan in the wild (Walsh *et al.*, 2002). In comparison to bigfin reef squid, a shorter exposure period was chosen for pygmy squid due to their brief lifespans, and given that mature animals were used, individuals were only expected to live a maximum of 30 (females) to 45 (males) days post-capture (Jackson, 1988).

Maximal and routine oxygen uptake measurements

Intermittent-flow respirometry (Clark *et al.*, 2013) was used to determine both routine oxygen uptake (routine $\dot{M}O_2$) and maximal oxygen uptake ($\dot{M}O_{2Max}$) for both cephalopod species. The $\dot{M}O_{2Max}$ was established by using a standard exercise challenge immediately before placing the animal in the respirometry chamber. The use of a swim chamber would not have proved effective in determining $\dot{M}O_{2Max}$ for either species. Pygmy squid tend to attach to the sides of the chamber with their unique adhesive mantle gland rather than swimming against the flow within a swimming chamber. Bigfin reef squid can unpredictably switch locomotory modes from fin undulations to jet propulsion, which can result in self-injury if they propel themselves against the chamber while swimming actively. Therefore, to achieve $\dot{M}O_{2Max}$ for pygmy squid, animals were chased with a dip-net in a small circular tank for 3 minutes, followed by a 15 second air exposure period (Roche *et al.*, 2013; Rummer *et al.*, 2016) immediately prior to introduction to the respirometry chamber. Pygmy squid (all individuals) were unable to continue jet escaping the dip-net before the end of the 3-minute

period due to exhaustion. The number of jets and ink discharges from each animal during the chase period were recorded. For the bigfin reef squid, however, due to potential injury reasons mentioned above, a different method was used to elicit $\dot{M}O_{2Max}$. Instead, bigfin reef squid were held in a large dip-net and periodically lifted in and out of the water (10 seconds in / 5 seconds out) for 3 minutes. On re-emersions into the water, squid would attempt to jet escape 0-4 times within the safety of the net. All squid ceased jetting before the end of the 3 minutes of periodic emersions. Bigfin reef squid were then subjected to a further 30-second air exposure period before being placed into respirometry chambers. The number of jets and ink discharges were also recorded.

The measurement period for intermittent-flow respirometry (time during which the flush pump was off) was determined as the minimum time required to ensure a steady slope representing the decline in O_2 concentration of the chamber down to 75-80% air saturation over time (O_2 uptake rate of the squid). The flush period (time during which the flush pump was on) was sufficient time for the O_2 concentration of the chambers to be sufficiently replenished back to ~100%. Pygmy squid were tested in 20 ml chambers submerged in an aquarium with continuous delivery of water from their CO_2 treatment system, with both flushing and recirculation pumps submerged and providing flow at 21.6 l h^{-1}. Preliminary experiments determined that routine $\dot{M}O_2$ of pygmy squid was reached in under 2 hours, so total trial time was set at 4 hours. The measurement period was set at 150 seconds followed by a 60 second flushing period. Bigfin reef squid were tested in 6,840 ml chambers also submerged in continuously replenished water from the CO_2 treatment system of the squid being tested. The flush and recirculation pumps for these chambers delivered water at 400 l h^{-1}. Measurement periods for bigfin reef squid lasted 85 seconds, followed by a 300-second

flush period; this cycle continued for a total 22-hour trial period. The squid to chamber volume ratio was between 1:20 and 1:50 for all animals and the O_2 concentration did not fall below 80% air saturation during measurement periods in any of the chambers for either species (Svendsen *et al.*, 2016).

Temperature-compensated O_2 concentration was continuously recorded (0.5Hz) using oxygen-sensitive REDFLASH dye on contactless spots (2 mm) adhered to the inside of a cut glass pipette tube set within the recirculation pump loop and linked to a Firesting Optical Oxygen Meter (Pyro Science e.K., Aachen, Germany) via fibre-optic cables. Data were analysed in LabChart version 8.1.3 (ADInstruments, Colorado Springs, CO, USA), and $\dot{M}O_2$ (in milligrams O_2 per kilogram of animal per hour) was calculated as the slope of the linear regression of oxygen concentration decline over time during the measurement period using the following equation:

$$\dot{M}O_2 = SV_{resp}M^{-1},$$

where S is the slope (in milligrams of O_2 per litre per second), V_{resp} is the volume of the respirometer minus the volume of the squid (in litres), and M is the mass of the squid (in kilograms). The volume of respirometry chambers included the volume of the chamber as well as that of the recirculation tubing and pump. The value of routine $\dot{M}O_2$ was calculated by taking the average of the lowest 10% of $\dot{M}O_2$, minus the background O_2 uptake, which was measured before and after each trial (assumed linear) (Rummer *et al.*, 2016). The $\dot{M}O_{2Max}$ was calculated by isolating the first five slopes into segments (each segment being 20% of the total measurement period) and selecting the highest rate of change found therein, and ensuring that the R^2 of slopes was above 0.95. Aerobic scope for each individual was calculated by subtracting the routine $\dot{M}O_2$ value from the $\dot{M}O_{2Max}$ value. Recovery time was defined as the

amount of time – from introduction to the chamber – for $\dot{M}O_2$ to first reach an equal or lesser value of the routine $\dot{M}O_2$ value for that individual. To restrict background respiration to <5% of a squid's routine $\dot{M}O_2$, chambers and pumps were rinsed with fresh water and 10% bleach solution after each trial and left to dry for 12 hours before being used again.

Statistical analyses

Statistical analyses were performed with R statistical software (R Development Core Team, 2018). Generalised linear mixed models (GLMM) with Gaussian distributions were used to compare response variables (routine $\dot{M}O_2$, $\dot{M}O_{2Max}$, aerobic scope, and recovery time) for pygmy squid between CO_2 treatments, with the number of jets and inks included as fixed factors and holding tank included as a random effect. The number of jets and inks were not included as fixed factors in models for routine $\dot{M}O_2$. Generalised linear models (GLM) with Gaussian distributions were used to compare the same response variables of bigfin reef squid between CO_2 treatments, with number of jets included as a fixed factor except in models for routine $\dot{M}O_2$. A tank effect was not applicable for bigfin reef squid as they were always housed individually; number of inks were excluded from models for bigfin reef squid as including it as a fixed factor violated assumptions of homogeneity of variance and/or normal linear relationships. The number of jets during the exercise protocol of bigfin reef squid included in the analyses ranged from 15-33. One animal performed only 11 jets and the resulting $\dot{M}O_{2Max}$ value, as well as the corresponding aerobic scope and recovery time values, were low outliers. Data from this individual was excluded as it is likely a true $\dot{M}O_{2Max}$ was not achieved. Residual analysis indicated that data met the assumptions of normality and homogeneity of variance.

2.4 – Results

Pygmy squid

Elevated CO_2 did not affect any of the traits measured for pygmy squid (**Table S1**). Pygmy squid had an average $\dot{M}O_{2Max}$ of 1,164 mg kg^{-1} h^{-1} under control and 1,117 mg kg^{-1} h^{-1} under elevated CO_2 (χ^2=0.112, p=0.738) conditions. The routine $\dot{M}O_2$ under elevated CO_2 was 18% lower under elevated CO_2 when compared to control conditions (341 mg kg^{-1} h^{-1} and 418 mg kg^{-1} h^{-1} respectively) but was not significantly different (χ^2=1.883, p=0.170) (**Fig. 2.1**). The aerobic scope of pygmy squid was similar between CO_2 treatments, with an average of 746 mg kg^{-1} h^{-1} at control compared to 776 mg kg^{-1} h^{-1} under elevated CO_2 (χ^2=0.929, p=0.335) (**Fig. 2.2**). Recovery time of pygmy squid under both control and elevated CO_2 conditions lasted for just under one hour, averaging 55 minutes and 12 seconds under control CO_2 and 53 minutes and 9 seconds under elevated CO_2 conditions (χ^2=0.006, p=0.936) (**Fig. 2.3**).

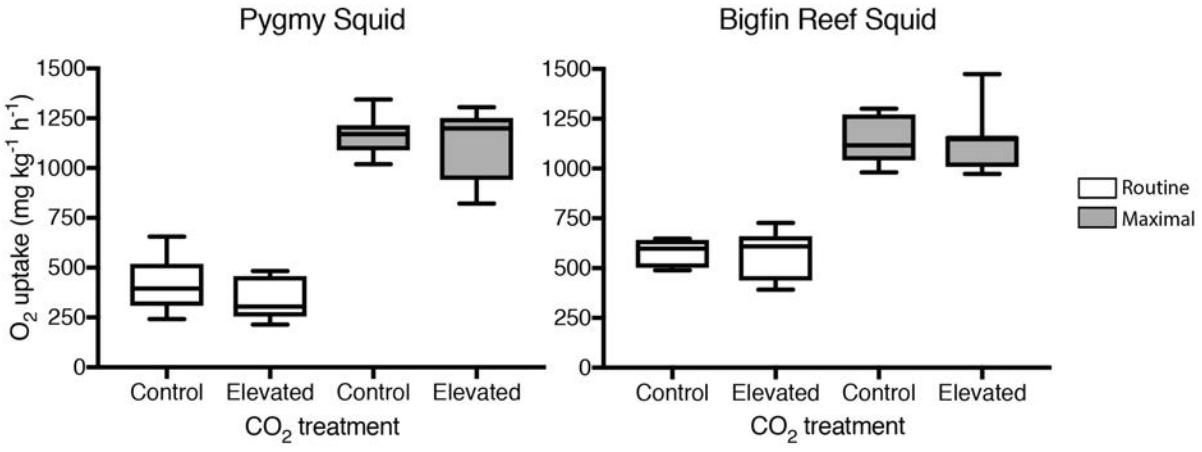

Figure 2.1 – Routine and maximal oxygen uptake – Boxplots (median and interquartile range with minimum and maximum of data) of the measured oxygen uptake of pygmy squid and bigfin reef squid at routine activity (white) and upon maximal exhaustion (grey) under current day control and elevated CO_2 treatment levels.

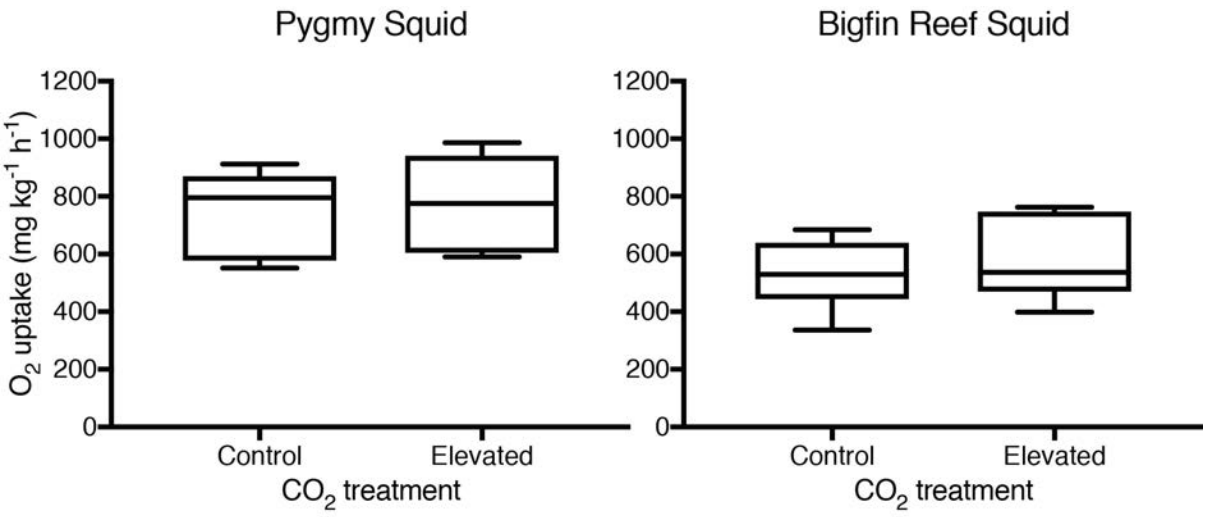

Figure 2.2 – Absolute aerobic scope – Boxplots (median and interquartile range with minimum and maximum of data) of the aerobic scope of pygmy squid and bigfin reef squid under current day control and elevated CO_2 treatment levels.

Bigfin reef squid

As observed in pygmy squid, elevated CO_2 did not affect any of the traits measured for bigfin reef squid (**Table S1**). The average $\dot{M}O_{2Max}$ of bigfin reef squid under control and elevated CO_2 treatment levels were almost identical at 1,138 mg kg^{-1} h^{-1} and 1,139 mg kg^{-1} h^{-1} respectively (χ^2=0.012, p=0.915). Average routine $\dot{M}O_2$ measurements between treatments were also similar at 579 mg kg^{-1} h^{-1} in the control and 565 mg kg^{-1} h^{-1} in elevated CO_2 (χ^2=0.084, p=0.772) (**Fig. 2.1**). The aerobic scope of bigfin reef squid averaged 526 mg kg^{-1} h^{-1} under control and 574 mg kg^{-1} h^{-1} under elevated CO_2 conditions (χ^2=0.610, p=0.436) (**Fig. 2.2**). Recovery time in bigfin reef squid was very similar between treatments, lasting an average of 8 hours and 8 minutes under control and 7 hours and 43 minutes under elevated CO_2 conditions (χ^2=0.083, p=0.774) (**Fig. 2.3**).

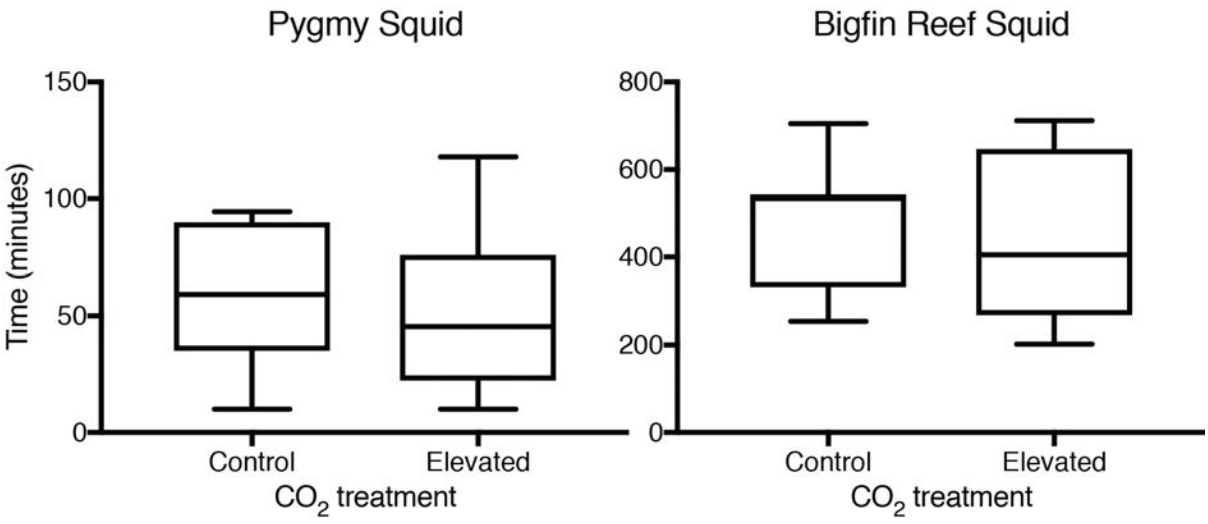

Figure 2.3 – Recovery time – Boxplots (median and interquartile range with minimum and maximum of data) of the time that pygmy squid and bigfin reef squid required to reach a stable routine oxygen uptake rate following exhaustive exercise under current day control and elevated CO_2 treatment levels.

2.5 – Discussion

After prolonged exposure to elevated CO_2 levels, I found no significant changes to the aerobic performance in either the two-toned pygmy squid (*I. pygmaeus*) or the bigfin reef squid (*S. lessoniana*) when compared with current-day control CO_2 conditions. The responses observed here under elevated CO_2 differ from the reduction in $\dot{M}O_{2Max}$ and routine $\dot{M}O_2$ observed in the jumbo squid (Rosa & Seibel, 2008) and the decrease in oxygen uptake rates observed in the common cuttlefish and European squid during late-stage embryo incubation (Rosa *et al*., 2014; Sigwart *et al.*, 2016). However, these results are consistent with those of juvenile common cuttlefish (Gutowska *et al.*, 2008) and experiments by Hu *et al*. (2014) in which bigfin reef squid showed no changes in routine $\dot{M}O_2$ under CO_2 levels of 1,585 µatm after a seven day exposure period. Furthermore, this study supports the models put forth by Birk *et al*. (2018), predicting that climate change relevant CO_2 levels would not alter the aerobic scope of squids. I predicted that elevated CO_2 should interfere with oxygen extraction causing a

decrease in the $\dot{M}O_{2Max}$, therefore reducing aerobic scope. However, the recent estimation that CO_2 levels of ~1,000 µatm would cause a drop in squid hemocyanin-O_2 saturation by no more than 1.6% (Birk *et al*., 2018) suggests that squid may be able to cope with elevated CO_2 without cost to their oxygen uptake capabilities. These results indicate that the respiratory physiology of the two species of tropical cephalopod studied here are likely to be resilient to realistic future CO_2 levels in the habitats they currently occupy.

Based on results from this study and those on other cephalopod species, it appears that elevated CO_2 elicits a range of aerobic responses in cephalopods. Reduced embryonic oxygen uptake in some cephalopods at elevated CO_2 (Rosa *et al*., 2014; Sigwart *et al.*, 2016) might be expected, as acid-base regulatory mechanisms in cephalopods often remain rudimentary until gills have formed and respiration switches from cutaneous (via skin) to branchial (via gills) (Hu *et al*., 2011a). Although little is known about the acid-base regulatory capabilities of pygmy squid, those of bigfin reef squid have been thoroughly investigated. Bigfin reef squid, among other cephalopods, have evolved ion regulatory epithelia in both the gills (Hu *et al.*, 2011b, 2014) and skin cells (Hu *et al.*, 2011b, 2013), which are effective in coping with acid-base disturbances. Acid-base regulatory abilities are an indispensable trait in cephalopods, as well as in all animals, as there is a continuous natural confrontation with respiratory CO_2 that can cause extra- and intra-cellular pH disturbances (Robertson, 1949; Hu *et al.*, 2013). Some cephalopods have advanced acid-base regulatory machinery, comparable to that of fishes, and can effectively regulate their acid-base balance at high levels of CO_2 without compromising aerobic capacities (Gutowska *et al.*, 2008, 2010). It appears that this may also be the case for both pygmy squid and bigfin reef squid, as $\dot{M}O_2$ remains unchanged at CO_2 levels (~1,000 µatm) projected for the end of the century under the business as usual CO_2

emissions scenario. However, there may still be energetic costs at much higher CO_2 levels, because the extreme CO_2 treatment (4,134 µatm) used by Hu *et al*. (2014) resulted in a 40% reduction in routine $\dot{M}O_2$ in bigfin reef squid.

The recovery times of pygmy squid and bigfin reef squid observed under control conditions were noticeably different between species. While pygmy squid recovered in an average time of less than one hour, bigfin reef squid took an average of approximately eight hours to return to their routine $\dot{M}O_2$. During trials, all pygmy squid were observed to use the adhesive gland on their mantle to remain attached to the wall of the respirometry chamber throughout the duration of the measurement period. In contrast, bigfin reef squid maintained a suspended position by the use of their undulating fins throughout the trials. This unique mechanism in pygmy squid grants the species a much greater capacity for rest than in the continuously swimming bigfin reef squid. This may help to explain why pygmy squid, under control conditions, overcame excess post-exercise oxygen consumption more rapidly. Furthermore, the routine $\dot{M}O_2$ values measured for pygmy squid are likely a more accurate representation of true standard metabolic rates, i.e. maintenance costs, than those of bigfin reef squid due to their mode of life.

Most squids are negatively buoyant, and the requirements of being 'at rest' while remaining suspended in the water column still have considerable aerobic costs (Bartol *et al.*, 2001). As bigfin reef squid recover from exercise, they remain relatively active in order to remain suspended in the water column. The species has large fins that run the full length of the mantle that can be used to maintain neutral buoyancy without the use of jetting. This allows them to spend, perhaps, a smaller fraction of their energy budget to maintain neutral

buoyancy when compared to other squid species with proportionally much smaller fins, requiring fin use to be coupled with jetting (Hu *et al.*, 2014). While this decoupled option of swimming with fin undulations alone in bigfin reef squid is more efficient than in more powerfully swimming pelagic squid species, pygmy squid can attach to a piece of seagrass or other benthic structure where they may truly rest, thus avoiding spending extra energy during recovery altogether. This mode of rest could be very beneficial to pygmy squid as lower mantle ventilation pressures in squids, such as during rest compared to during active swimming, lead to higher oxygen extraction rates (Melzner *et al*., 2006).

It is worth noting that the average values of routine $\dot{M}O_2$ in bigfin reef squid under control CO_2 levels observed by Hu *et al*. (2014) were markedly higher, by about 70%, than those observed here. The routine $\dot{M}O_2$ values in this experiment were determined after a 22-hour trial period; whereas, the previous experiment determined routine $\dot{M}O_2$ from a 20-30 minute measurement period. The results from this experiment indicate that bigfin reef squid experience excess post-exercise oxygen consumption for approximately eight hours after exhaustive exercise. While Hu *et al*. (2014) did not exercise the squid in their experiments, it is likely that a measurement period of 20-30 minutes did not allow for a true routine $\dot{M}O_2$ measurement due to the handling stress and introduction to the respirometry chamber. Nevertheless, considering all animals were handled and introduced to the chambers in the same way, the comparisons among CO_2 treatments from the previous experiment are still useful. Furthermore, there were comparable effects of similar elevated CO_2 levels, causing unaltered $\dot{M}O_2$ in both the former experiments and in those which were performed here.

Results from this study contribute to the increasing evidence suggesting variation in tolerance to elevated CO_2 in cephalopods among species and among the traits being tested. It has been

suggested that the active, high-energetic lifestyle, along with occurrences of natural hypercapnia during the course of embryonic development, constitute factors that pre-adapt cephalopods to more easily cope with future elevated CO_2 levels (Melzner *et al.*, 2009b). While elevated CO_2 has been shown to illicit negative physiological responses in some cephalopod species (e.g., Kaplan *et al.*, 2013; Sigwart *et al.*, 2016), other studies have observed no adverse effects of elevated CO_2 on the same traits in other species (e.g., Gutowska *et al.*, 2010; Birk *et al.*, 2018). Physiological stress from elevated CO_2 during the early ontogeny of animals has been described as the "true bottleneck" through which species must pass in order to successfully tolerate future elevated CO_2 oceans, even for species that demonstrate tolerance in other traits (Melzner *et al.*, 2009b). Therefore, it will be important for future studies to investigate the effects of elevated CO_2 on cephalopods during their embryonic and paralarval development, especially in species that show no adverse effects of elevated CO_2 in other physiological traits as adults and such as those studied here.

This study has demonstrated that the routine $\dot{M}O_2$, $\dot{M}O_{2Max}$, aerobic scope, and recovery time of two tropical cephalopod species are unaltered following long-term exposure to elevated CO_2. In warmer tropical latitudes, where the energy requirements of cephalopods are higher, altered aerobic performance could be particularly consequential. However, it appears that CO_2 levels projected for the end of this century will not adversely affect the respiratory performance of either the two-toned pygmy squid or bigfin reef squid. The unaltered aerobic performance of these ecologically distinct cephalopod species, from separate taxonomic orders, after long-term exposure to elevated CO_2 indicates that a wide range of cephalopod species may have the aerobic capacity to cope with an increasingly CO_2-rich ocean. However, as some previous studies on cephalopods have observed negative effects of elevated CO_2 on

respiratory performance, the results demonstrated here indicate the importance on understanding the potentially species-specific effects, which can have critical implications for the structure of marine ecosystems in the future.

Chapter 3: Elevated carbon dioxide affects reproduction and embryonic development in the pygmy squid, *Idiosepius pygmaeus*

Blake L. Spady, Philip L. Munday, Sue-Ann Watson

3.1 – Summary

The ocean is absorbing anthropogenic carbon dioxide (CO_2) from the atmosphere and projected future levels of pCO_2 could have negative implications for many marine organisms. The effects of elevated CO_2 on the life-history characteristics of cephalopods has been little studied, but they appear to be most vulnerable during the embryonic period. The potential effects of parental exposure to elevated CO_2 on cephalopod reproduction and embryonic development are unknown. In this study, adult two-toned pygmy squid (*Idiosepius pygmaeus*) were paired in ambient control or elevated CO_2 concentrations and allowed to breed. The reproductive characteristics of parental pairs and the developmental characteristics of their eggs were compared between treatments. Parents maintained in elevated CO_2 produced clutches with 40% fewer eggs compared with parents in control conditions. There was no difference in initial egg size between treatments, but in the final stages of development after embryonic respiration had caused eggs to swell, egg area in elevated CO_2 were 8% larger compared with control conditions. Embryo area, measured directly after spawning, was 14% smaller in the elevated CO_2 treatments compared with embryos in control conditions. Embryos at elevated CO_2 remained smaller than those at control throughout development and were 5% smaller upon hatching. There were no effects of elevated CO_2 on fertility, time to hatch, or hatching success. Lastly, adult female pygmy squid maintained at elevated CO_2 laid eggs 40% closer together than those in control conditions, indicating a possible effect of

elevated CO_2 on reproductive behaviour. These results show that elevated CO_2 can affect reproduction and embryonic development of the two-toned pygmy squid, leading to fewer and smaller hatchlings. As the potential for adaptation is influenced by reproductive success, this could influence their capacity to cope with future ocean conditions.

3.2 – Introduction

Since the industrial revolution, atmospheric carbon dioxide (CO_2) concentrations have increased by over 40% (Dlugokencky & Tans, 2018), and are now higher than any time in the past 800,000 years (Lüthi *et al.*, 2008). If the current emissions trajectory continues, it is projected that atmospheric CO_2 could exceed 900 ppm by the end of this century (Collins *et al.*, 2013). As the partial pressure of CO_2 (pCO_2) at the ocean surface is in approximate gas equilibrium with the atmosphere, pCO_2 in the ocean is rising at the same rate as the atmosphere (Doney, 2010). Along with the higher CO_2 concentrations, it is projected that seasonal fluctuations in ocean pCO_2 will be amplified in the future, due to the increased Revelle (buffer) factor of acidified seawater (McNeil & Sasse, 2016; Gallego *et al.*, 2018). Consequently, marine organisms could already experience CO_2 levels greater than 1,000 µatm for several months each year by 2080 (McNeil & Sasse, 2016). The rapid rate of these changes to ocean chemistry could put many marine species and ecosystems at risk (Hoegh-Guldberg *et al.*, 2007; Kroeker *et al.*, 2013; Haigh *et al.*, 2015; Cattano *et al.*, 2018).

While elevated CO_2 causes a range of physiological effects on marine animals (Pörtner *et al.*, 2004; Doney *et al.*, 2009), it has been suggested that earlier developmental stages and reproductive periods of marine animals may be more sensitive to the effects of elevated CO_2

than later in development (Ishimatsu *et al.*, 2004; Melzner *et al.*, 2009b; Kroeker *et al.*, 2010). Consistent with this hypothesis, a meta-analysis of 228 studies comparing the effects of elevated CO_2 among taxa found some of the most common negative effects were reduced survival, growth, and development during the early life history (Kroeker *et al.*, 2013). Furthermore, the negative effects of elevated CO_2 on early life-history traits are particularly prevalent in molluscs, with mean reductions in survival by 34%, growth by 17%, and development by 25% (Kroeker *et al.*, 2013). Another meta-analysis by Parker *et al*. (2013) focusing specifically on molluscs, found that in 12 bivalve species and 5 gastropod species, all but one species responded negatively to elevated CO_2 in relation to their embryonic and larval development. Some of the more common negative responses to elevated CO_2 included reduced survival, growth and development, as well as adverse effects on reproduction (Parker *et al.*, 2013).

The effects of elevated CO_2 on reproduction in molluscs include reduced reproductive output and fertility (Parker *et al.*, 2009, 2010; Kimura *et al.*, 2011). Fertilisation in marine invertebrates was originally predicted to be the most sensitive stage to elevated CO_2 (Kurihara, 2008; Parker *et al.*, 2013) as sperm motility is suppressed in more acidic seawater (Christen *et al.*, 1983). However, effects of elevated CO_2 on fertilisation in molluscs is varied (e.g., Parker *et al.*, 2010; Bechmann *et al.*, 2011), with many species responding negatively while others are not affected (Parker *et al.*, 2013). Reduced reproductive output resulting from elevated CO_2 may be explained by changes in energy allocation due to increased maintenance costs (Dupont & Thorndyke, 2009). Many organisms also adjust their reproductive output based on environmental conditions (Hughes, 2017), and reduced egg production at elevated CO_2 could be an example of this. However, most of the studies on the

effects of elevated CO_2 on the reproduction and early life history traits in molluscs have involved highly calcifying species, such as gastropods and bivalves, while other molluscs such as cephalopods have not been as thoroughly investigated (Parker *et al.*, 2013).

Elevated environmental CO_2 can be stressful for cephalopod embryos as the abiotic conditions inside the egg are directly influenced by the pCO_2 of the surrounding seawater (Hu *et al.*, 2011a). During the final stages of cephalopod embryonic development, they are exposed to very low pO_2 values due to increased metabolic rates and the egg casing acting as a diffusion barrier for dissolved gases (Cronin & Seymour, 2000). This not only decreases pO_2 inside the egg, but increases pCO_2 as well (Gutowska & Melzner, 2009; Long *et al.*, 2016). Therefore, elevated environmental CO_2 is a stressor to cephalopod development (Hu *et al.*, 2011a), because an increase of pCO_2 in the perivitelline fluid within the egg is needed to conserve the diffusion gradient of CO_2 between the egg and the environment (Dorey *et al.*, 2013).

While the effects of elevated CO_2 on the reproductive traits of cephalopods are unknown, previous studies have reported effects on early life-stages. Atlantic longfin squid (*Doryteuthis pealeii*) reared from eggs to hatchlings demonstrated developmental changes under high CO_2 conditions (1,300 and 2,200 µatm), including increased time to hatching, shorter mantle lengths, as well as reduced surface area and abnormally shaped aragonite statoliths, which are crucial for orientation and motion detection (Kaplan *et al.*, 2013; Zakroff, 2013). The bigfin reef squid, *Sepioteuthis lessoniana*, had reduced late-stage embryo mass at very high CO_2 (~4,600 µatm) compared to control conditions (Hu *et al.*, 2013). The common cuttlefish, *Sepia officinalis*, exhibited reduced mantle length and body mass in embryos and delayed hatching

at very high CO_2 (~3,600 µatm), but not at less extreme levels (1,300-1,500 µatm) (Hu *et al.*, 2011a; Dorey *et al.*, 2013; Sigwart *et al.*, 2016). However, juveniles of the same species held at very high CO_2 (4,200-6,000 µatm) maintained calcification and had the same growth rates as those held at control (Gutowska *et al.*, 2008). Most of these adverse effects have been demonstrated at CO_2 concentrations well above those predicted for the near future due to climate change. Effects of pCO_2 levels predicted for the ocean by the end of this century (e.g., 1,000 µatm) are uncertain. Furthermore, the potential for effects after parental exposure to elevated CO_2 in cephalopods has not been investigated.

In the present study, I aimed to determine if reproductive traits and embryonic development of a tropical cephalopod, the two-toned pygmy squid (*Idiosepius pygmaeus*), are affected by exposure to elevated CO_2. Spady *et al*., (**Chapter 2**) found that long-term exposure to elevated CO_2 had no effect on the aerobic scope of adult *I. pygmaeus*, therefore it is expected that the energy available for reproduction at elevated CO_2 will remain optimal. However, elevated CO_2 did increase activity levels and affect predator avoidance and predatory behaviours (Spady *et al.*, 2014, 2018, **Chapter 4**), therefore behavioural effects on reproduction could occur. I paired squid in elevated CO_2 and ambient control conditions and allowed them to breed. I measured clutch size, distance between eggs, percent of eggs that were fertile, time to hatch, hatching period, embryo size throughout development, and egg size. Furthermore, embryos were measured periodically throughout development to determine if any adverse effects occurred during late embryonic phases, when CO_2 levels inside the egg are naturally higher due to increased respiration, or earlier in development, possibly as a result of parental exposure to elevated CO_2. I hypothesised *I. pygmaeus* eggs to show no significant differences between treatments in embryo or egg sizes in the very early stages of development, but

rather that later stages would be affected by elevated CO_2 as a result of increased pCO_2 within the egg capsule.

3.3 – Methods

Study species

The species used in this study, the two-toned pygmy squid (*Idiosepius pygmaeus*), achieves a maximum mantle length of ~20 mm and has a generation time of ~90 days (Jereb & Roper, 2005). Males mature as small as 6.8 mm and as young as 42 days, whereas females are around 13 mm and 60 days old at maturity (Jackson, 1988). Individuals are able to reproduce multiple times throughout their adult life and females produce an average of 640 eggs in 11 clutches over an 18 day period (Lewis & Choat, 1993). Eggs are laid in a gelatinous mass and attached to seagrass or substrate. *I. pygmaeus* occupies nearshore waters and can often be found in seagrass habitats (Nishiguchi *et al*., 2014) and their observed prey in the field is the sergestid shrimp, *Acetes sibogae australis*.

CO_2 treatment systems

Experiments were conducted using 4 x 8,000 L recirculating seawater systems at James Cook University's research aquarium in Townsville, Australia. CO_2 treatments were duplicated and set at a current-day control (442-451 µatm) and an upper end-of-century projection following RCP8.5 (high CO_2, 939-944 µatm) (Collins *et al.*, 2013). A custom-built pH control system dosed CO_2 into 3,000 L sumps to achieve the desired pH level in the elevated CO_2 treatment. Briefly, pH was measured continuously by an inline ISFET pH sensor (Tophit CPS471D, Endress+Hauser, Reinach, Switzerland) that communicated with a computerised controller (OMNI C40 BEMS, Innotech, Brisbane, Australia) and regulated the CO_2 dosing.

pH_{NBS} was measured daily (Seven2Go Pro, Mettler Toledo, Switzerland) and dosing set points were adjusted as necessary to maintain the target pCO_2 in each treatment. Equilibrated seawater from each system was delivered at a rate of 1.5 L min^{-1} to aquaria containing squid. Temperature was measured daily in each tank (Comark C26, Norfolk, UK).

Water samples were taken weekly to determine pH on the total scale (pH_T) by spectrophotometry (UVmini-1240, Shimadzu, Suzhou Instruments Co. Ltd., Kyoto, Japan) using m-cresol purple as an indicator dye (Dickson & Millero, 1987; Dickson *et al.*, 2007). Total alkalinity was estimated weekly by Gran Titration (888 Titrando Metrohm AG, Switzerland) ensuring titration calibrations remained within one percent of certified reference material from Dr. A.G. Dickson (Scripps Institution of Oceanography, batch #135). Salinity was measured weekly using a conductivity sensor (HQ15d, Hach, Loveland, CO, USA). Carbonate chemistry parameters (**Table 3.1**) were calculated in CO2SYS (Pierrot *et al.*, 2006) using the constants K1, K2 from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and Dickson *et al.* (2007) for $KHSO_4$.

Table 3.1 – Seawater carbonate chemistry – The temperature, salinity, $pH_{(T)}$, total alkalinity, and pCO_2 for duplicated control and elevated CO_2 treatments. Values are means ± SD.

Treatment	Temperature (˚C)	Salinity	$pH_{(T)}$	Total alkalinity (µmol kg^{-1} SW)	pCO_2 (µatm)
Control-1	28.5 (±0.1)	35.7 (±0.4)	8.05 (±0.03)	2632 (±79)	451 (±41)
Control-2	28.4 (±0.2)	35.7 (±0.6)	8.04 (±0.03)	2576 (±37)	442 (±29)
Elevated CO_2-1	28.4 (±0.1)	35.4 (±0.8)	7.78 (±0.01)	2617 (±72)	939 (±44)
Elevated-CO_2-2	28.5 (±0.2)	36.0 (±0.4)	7.77 (±0.01)	2557 (±49)	944 (±33)

Animal care and egg measurements

Two-toned pygmy squid were collected by dip net (500 µm mesh) in September 2017 from Cleveland Bay in Townsville, Queensland, Australia (19˚24'S, 146˚82'E) and immediately transported to the experimental facility. Squid were kept in aquarium fish breeder baskets (155 x 135 x 150 mm, 900 µm mesh) within larger tanks (40 x 30 x 30 cm) at a maximum of one pair per tank. Each breeder basket had fresh supply of treatment seawater flowing directly into the basket. Animals were provided with cut PVC structures for shelter and as a spawning substrate. Squid were maintained at control conditions for 24-hours in separate tanks before being transferred to their CO_2 treatment, where they remained for 14 days before being paired with a breeding partner from the same CO_2 treatment. This exposure period accounts for >45% of the adult lifespan of female *I. pygmaeus* (Jackson, 1988). Six parental pairs were kept at each CO_2 treatment. The wet-mass of individuals from parental pairs were recorded after the 14-day CO_2 treatment period and prior to pairing. There was no apparent effect of handling and measuring parental pairs on their breeding based on preliminary observations of reproductive output in separate pairs. Squid were fed glass shrimp (*Acetes sibogae australis*) three times daily.

Baskets were checked for eggs four times daily, beginning 24 hours after individuals were paired together. Some eggs were attached to the provided PVC pipes during spawning while others were attached to the netting of breeder baskets. When eggs were found, after ensuring that the female had finished laying the clutch, they were immediately counted and photographed from above at 2x and 3.13x magnification with a stereoscopic microscope (Olympus SZX7) and then placed back in the same tank but separated from their parental pair in a separate breeder basket. Egg clutches were then photographed (Olympus SC50) every 24

hours after first being discovered until all eggs had hatched. The first two egg clutches from each parental pair were analysed, totalling 12 clutches per CO_2 treatment.

Egg characteristics and developmental measurements were made using ImageJ® software from digital photographs taken of egg clutches. Egg area and space between eggs were measured immediately after spawning. The space between eggs was quantified as the shortest distance between outer egg membranes from each individual egg to the nearest neighbouring egg. Embryo developmental stages were identified following Yamamoto (1988). Embryo measurements (to the nearest $\pm$ 1 µm) were made during stage 1 (directly after spawning), stage 23 (a distinct stage in which retinas and fins become apparent), and stage 30 (the final stage where the yolk mass is almost exhausted and embryos are ready to hatch). Different metrics were used to measure embryo size at different stages of development due to the major changes in morphology throughout development: embryo area was measured at stage 1, head width at stage 23, mantle length at stage 30. Each metric is a stage-appropriate indicator of animal size. Embryo area at stage 1 was estimated as the area within the outer perimeter of the embryo. At stage 23, the width of the head was measured as the distance between eyes. The width of the head could not be measured in some embryos due to their position within the egg. Nevertheless, more than half of the embryos from each clutch were able to be measured during this stage. At stage 30, embryo mantle length was measured from the posterior end of the mantle to the funnel. Due to the position of embryos within the egg some individuals at stage 30 could not be measured as well, however, more than 85% of embryos from each clutch were measured during this stage. For all other metrics, the entire clutch was sampled.

Time to hatch was defined as the time from spawning until 25% of eggs within the clutch had hatched because the hatching period extended over several days. Hatching period was measured as the time from the first individual to hatch to the last individual to hatch. Embryo deformations were counted after stage 23 and determined by obvious morphological mutations such as drastically undersized mantles relative to head size. Lastly, the number of animals to hatch was determined after there had been no more hatching for 48 hours. Embryos remaining in eggs after this period were considered to not have hatched.

Statistical analyses

Data were analysed using R statistical software (R Development Core Team, 2018). Reproductive characteristics were compared between treatments using generalised linear models (GLM). CO_2 treatment, maternal and paternal mass, clutch number (whether first or second clutch), and spawning substrate were included as fixed factors. Preliminary analyses showed that replicate CO_2 treatments had no effect in any of the models, so the two control systems were grouped together, as were the two elevated CO_2 systems. Models with count data (clutch size, number of deformed eggs, days to hatch, and hatching period) used negative binomial distributions. Percent of fertile eggs and percentage of eggs to hatch data were analysed with logit transformations in gaussian distributions. Distance between eggs data was zero inflated (as eggs that were touching each other were measured with a distance of 0) so were analysed with a quasipoisson distribution. Models for embryo size in stages 1 and 23 used a beta regression as these values were continuous between 0 and 1. Mantle length at hatching data were normal and met assumptions under a gaussian distribution. The effects of elevated CO_2 on the egg area between developmental stages were analysed with a repeated measures generalised linear mixed effects model (GLMM) with a Gamma

distribution and compared with a post-hoc Tukey's HSD test. Here, CO_2 treatment and developmental stage were included as fixed factors along with maternal and paternal mass, spawning substrate and clutch number, while clutch ID was included as a random effect.

3.4 – Results

Mean clutch size was >40% smaller in parental pairs held at elevated CO_2 compared to control (χ^2=8.096, p=0.004). Control animals laid a mean of 74 eggs per clutch, whereas animals that bred at elevated CO_2 laid a mean of 44 eggs per clutch (**Fig. 3.1a**). Eggs were laid significantly closer together, by 41%, at elevated CO_2 compared to control (χ^2=30.925, p<0.001) (**Fig. 3.1b**). There was no difference in the mean percentage of fertile eggs per clutch (χ^2=1.975, p=0.160) (**Fig. 3.1c**). Two parental pairs held at elevated CO_2 were strong outliers, producing clutches with only 2-5% of fertilised eggs, while the remaining clutches at elevated CO_2 contained between 90-100% fertile eggs. There was no difference in the hatching success of fertilised eggs (**Fig. 3.2a**), which averaged 96% for both treatments (χ^2=0.004, p=0.947). There were also no differences in the time to hatch (χ^2=0.091, p=0.763) (**Fig. 3.2b**), or in the hatching period (χ^2=0.196, p=0.658) (**Fig. 3.2c**).

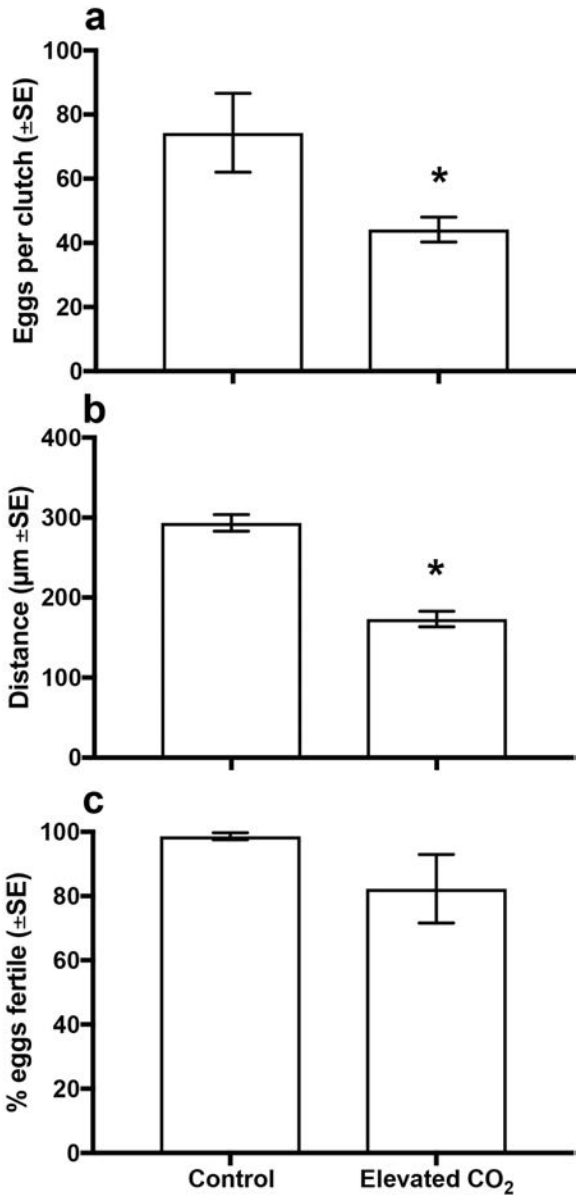

Figure 3.1 – Reproduction and spawning – Effects of elevated CO_2 compared to control on the (a) number of eggs per clutch, (b) distance between eggs within each clutch, (c) percent of fertile eggs per clutch. All values are means $\pm$ SE from each treatment. Asterisks indicate a significant difference from control.

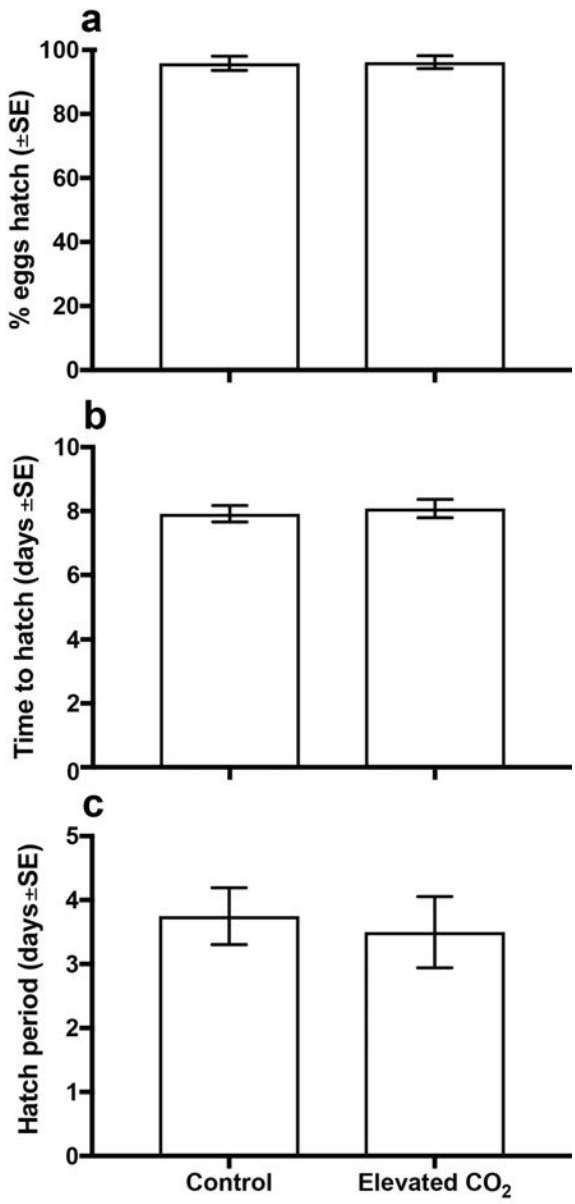

Figure 3.2 – Hatching success – Effects of elevated CO_2 compared to control on the (a) percent of fertile eggs to successfully hatch, (b) time to hatch from spawning, (c) time for all eggs to hatch from beginning of hatching period. All values are means ± SE from each treatment.

Embryos in the elevated CO_2 treatment were nearly 14% smaller than those in the control treatment (χ^2=79.786, p<0.001) immediately after spawning (stage 1). Embryos of elevated CO_2 parents had an average area of 466 µm^2 compared to 403 µm^2 for embryos of control parents (**Fig. 3.3a**). The width of the head at stage 23 of embryonic development was also significantly smaller in the elevated CO_2 treatment compared to embryos in control

conditions (χ^2=24.940, p<0.001) (**Fig. 3.3b**). Mantle length (ML) at stage 30 was also significantly smaller in elevated CO_2 compared with embryos in control conditions (χ^2=39.040, p<0.001), although the difference was only 5% of ML (**Fig. 3.3c**).

Egg size immediately after spawning, was not significantly different between treatments immediately after being spawned (Tukey's HSD: p=0.913). By the final stage of embryonic development eggs had swollen and egg size was significantly larger in both treatments (χ^2=602.916, p<0.001). However, the amount of egg swelling differed between treatments (Tukey's HSD: p=0.009); eggs in the high CO_2 treatment had expanded by 32%, whereas eggs in control conditions swelled by only 24% (**Fig. 3.4**).

Deformations of embryos occurred in both treatment groups but were extremely uncommon and not more prevalent in either group (χ^2=0.365, p=0.546). The spawning substrate chosen correlated with embryo size (χ^2=33.556, p<0.001) and egg size (χ^2=86.565, p<0.001), with those laid on PVC pipe being larger than those on the basket netting. Stage 30 ML was significantly smaller (4.7%) in the second clutch from a parental pair compared to the first clutch (χ^2=31.557, p<0.001). Maternal mass had a significant positive relationship with hatchling ML (χ^2=42.158, p<0.001) and a significant negative relationship with the distance between eggs (χ^2=16.397, p<0.001) and egg size (χ^2=32.025, p<0.001). Paternal mass had a significant negative relationship on egg size (χ^2=17.157, p<0.001) (**Table S2**).

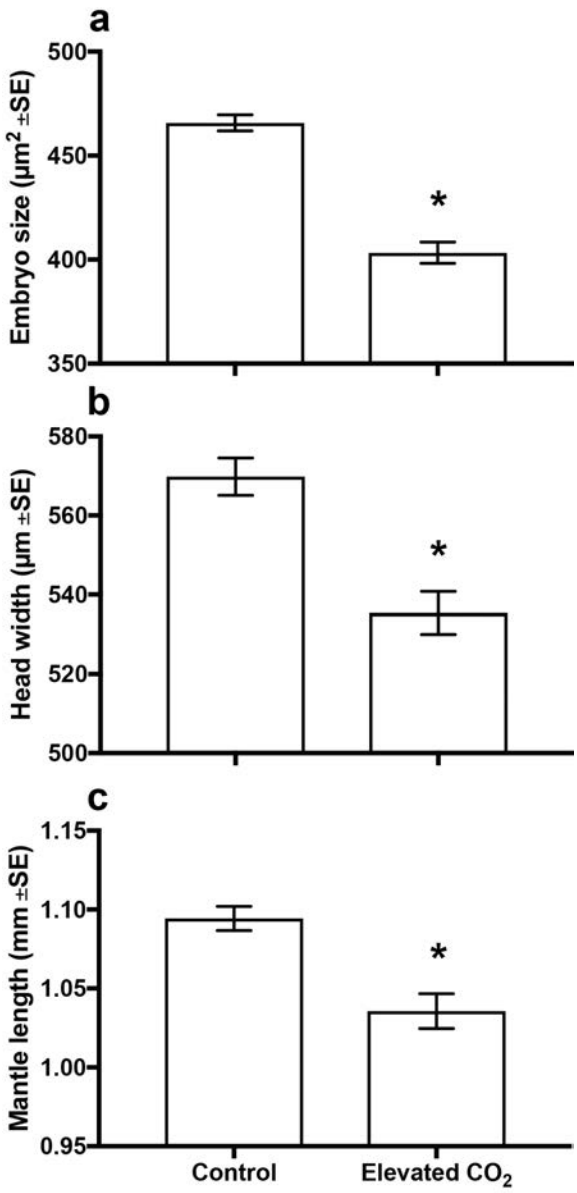

Figure 3.3 – Developmental traits – Effects of elevated CO_2 compared to control on the (a) embryo area upon spawning, (b) width of head at stage 23 of development, (c) mantle length at final stage (stage 30) of development. All values are means ± SE from each treatment. Asterisks indicate a significant difference from control.

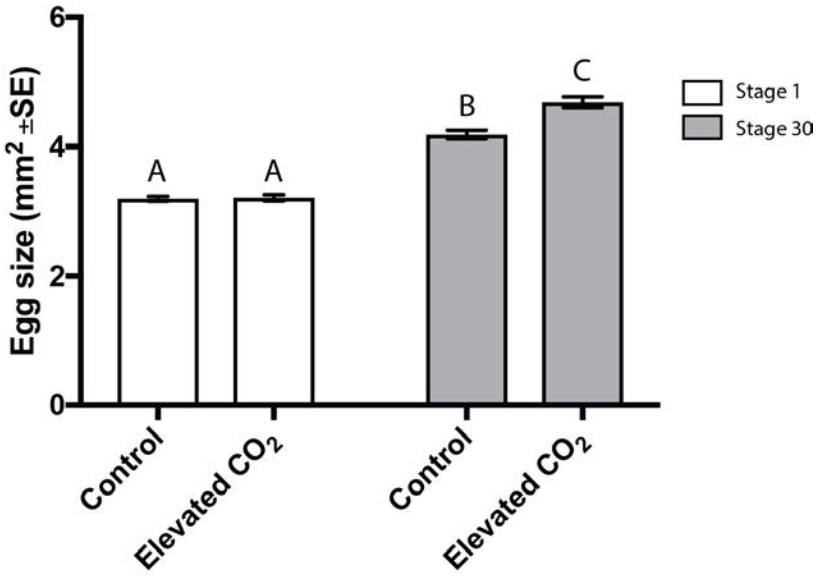

Figure 3.4 – Egg size and swelling – Effects of elevated CO_2 compared to control on the area of eggs at stage 1 (white bars) and after egg swelling at stage 30 (grey bars). All values are means ± SE from each treatment. Different letters represent significant differences between treatments.

3.5 – Discussion

Exposure of pygmy squid breeding pairs and their eggs to potential end of century CO_2 levels (~942 µatm) resulted in reduced reproductive output, inhibited development, as well as potential behavioural changes during spawning. Animals from the elevated CO_2 treatment laid 40% fewer eggs per clutch on average and eggs were laid closer together compared with those from control parents. The size of pygmy squid in the elevated CO_2 treatment were smaller than those spawned in control conditions and remained smaller until hatching. There was no effect of elevated CO_2 on egg size, percent of fertile eggs per clutch, percent of eggs that hatch, or in the time to hatch. These results show that elevated CO_2 levels that can occur periodically in coastal habitats, and will become more frequent in the future due to continued uptake of anthropogenic CO_2, can affect important reproductive and developmental traits in pygmy squid, with potential implications for their populations.

One possible explanation for the smaller clutch and embryo size at spawning is that elevated CO_2 could have altered the energy budget of adult *I. pygmaeus*. Elevated CO_2 did not increase routine $\dot{M}O_2$ in adult pygmy squid (**Chapter 2**), and thus their overall energetic demand appears unaffected by elevated CO_2. However, there could have been increased energetic costs associated with exposure to elevated CO_2, such as increased energy allocated to acid-base regulation (Gutowska *et al*., 2010), which was traded-off against energy allocated to reproduction. Alternatively, the smaller clutch sizes seen here could be an effort to allocate more energy towards individual embryos so they could cope with elevated CO_2. Embryos in the southern calamari squid (*Sepioteuthis australis*) develop significantly faster when in small egg masses compared to those from large egg masses (Steer *et al.*, 2003), although I did not observe that effect here.

Until relatively recently, practically all cephalopods were thought to be semelparous, meaning they reproduce only once during their lifetime, allocating all available energy into a single reproductive event with the trade-off of post-reproductive mortality (Rocha *et al.*, 2001). However, *I. pygmaeus* are iteroparous, meaning they can reproduce multiple times throughout their adult life, adjusting their reproductive effort based on current environmental conditions (Lewis & Choat, 1993; Rocha *et al.*, 2001; Hughes, 2017). A benefit of being iteroparous is that during environmental stress, individuals have the option of reducing their allocation of energy towards reproductive efforts, saving energy for future reproduction (Clutton-Brock, 1984; Hamel *et al.*, 2011). It is possible that the stress of elevated CO_2 exposure on adults in the current study has caused them to temporarily reduce the amount of energy allocated towards egg production, resulting in the reduction in clutch sizes as well as in initial sizes of embryos. Cephalopods are effective acid-base regulators (Hu

et al., 2011a, 2011b, 2013, 2014), however, the cost of this regulatory effort is not fully understood. Some cephalopods have advanced acid-base regulatory machinery, and can effectively regulate at elevated CO_2 conditions without affecting their energy budget to the extent of compromising aerobic capacities (Gutowska *et al.*, 2008, 2010). Investigations into the effects of elevated CO_2 on the aerobic scope of adult *I. pygmaeus* yielded no effect of elevated CO_2 (**Chapter 2**). This suggests that the aerobic scope of *I. pygmaeus* may not be a definitive metric of their performance.

A decrease in clutch sizes in response to elevated CO_2 could potentially have implications for population recruitment. The large population sizes and high rates of population increase of most cephalopod species gives them advantages over many species in competing for resources and occupying niches (Boyle & Rodhouse, 2005). If fewer eggs are being laid and population sizes decline, populations might become more sensitive to environmental changes. In this experiment, I only analysed the first two clutches from each pair and am therefore unaware if elevated CO_2 has an effect on the number of clutches produced. The damselfish, *Amphiprion percula*, produced up to 75% more clutches in breeding pairs that were exposed to elevated CO_2 (Welch & Munday, 2016). If *I. pygmaeus* shows a positive response in their number of clutches produced at elevated CO_2, this could offset the reduced number of eggs per clutch. Future studies should investigate the effects of elevated CO_2 on the full range of egg clutches that parents produce.

I found that the greatest difference was immediately after spawning. Embryos from parents in the elevated CO_2 treatment were on average 14% smaller than those of control parents after spawning, whereas the average size of embryos from elevated CO_2 during stage 23 was

only 6% less and then 5% less at the final stage of embryonic development. Stage 1 embryos were measured immediately after spawning, therefore, the initial differences in embryo size between CO_2 treatments must have been due to parental exposure to elevated CO_2. There was no difference in the time to hatch between treatments, but the relative size of embryos in the elevated CO_2 treatment compared to the controls was more pronounced immediately after spawning than at the end of development. This decrease in embryo size differences between CO_2 treatments in late stage development could be a compensatory effect resulting from the smaller embryo sizes at elevated CO_2. Stress experienced during early life can often be compensated for with more rapid growth rates and return to a normal growth trajectory (Metcalfe & Monaghan, 2001; Diederich *et al.*, 2011). However, there can be costs to compensatory growth later in life, such as decreased lifespan and altered age at sexual maturation (Metcalfe & Monaghan, 2001).

Parental exposure to elevated CO_2 has been linked to adverse effects on offspring in some marine invertebrates, such as reduced settlement success and juvenile survival (Dupont *et al.*, 2013). However, with more prolonged parental exposure, some of these adverse effects were ameliorated (Dupont *et al.*, 2013). Indeed, parental exposure to elevated CO_2 has been observed to reduce the severity of adverse effects of elevated CO_2 on offspring traits in a variety of marine species (Form & Riebesell, 2012; Miller *et al.*, 2012; Parker *et al.*, 2012, 2015; Allan *et al.*, 2014; Thor & Dupont, 2015; Stiasny *et al.*, 2018). Importantly, the 14 days duration of adult pygmy squid exposure to elevated CO_2 represents >45% of their adult lifespan, therefore, it seems unlikely that the negative effects on embryo size observed here would have been reversed by longer parental exposure to elevated CO_2.

Elevated CO_2 also appears to have altered female behaviour during spawning. Females from the elevated CO_2 treatment laid eggs that were 40% closer together on average compared to clutches from control parents. This could have direct effects on embryonic development. In many marine invertebrates, the structure and density of egg clutches is important in ensuring oxygen availability to the embryos (Lucas & Crisp, 1987; Lee & Strathmann, 1998; Lardies & Fernández, 2002). For example, embryos located on the periphery of clutches in the southern calamari squid, *S. australis*, develop faster and have lower levels of mortality than siblings located in the centre of clutches (Steer *et al.*, 2003). If *I. pygmaeus* embryos are affected by position within the clutch, similar to that of *S. australis*, increased crowding of eggs at elevated CO_2 could be a factor in the decreased hatchling size observed here. These crowded conditions in *I. pygmaeus* could be further compounded by the increased swelling of eggs at the later stages of development seen here in the elevated CO_2 treatment.

While there was no difference in the mean area of eggs upon spawning, at the final stage of development eggs from the elevated CO_2 treatment were significantly larger than those at control. The swelling of eggs is common in aquatic organisms, and leads to a reduction in the thickness of the egg wall, increasing gas conductance, and increasing the surface area available for gas exchange (Cronin & Seymour, 2000). This combats the natural high pCO_2 and low pO_2 conditions of the perivitelline fluid within cephalopod eggs (Gutowska & Melzner, 2009). However, with elevated environmental CO_2, *S. officinalis* demonstrates a further swelling of their eggs compared to at control conditions. Therefore, the increased swelling observed in pygmy squid eggs could be a positive response to elevated CO_2, as it would help increase the flux of CO_2 from the egg to the external environment (Lacoue-Labarthe *et al.*, 2011; Sigwart *et al.*, 2016). However, swelling in cephalopod eggs does not prevent pO_2 from

falling to critical levels or prevent pCO_2 from rising within the eggs (Cronin & Seymour, 2000; Rosa *et al.*, 2013). Furthermore, increased swelling coupled with the increased crowding of eggs upon spawning could cause more stagnant areas around the egg, potentially resulting in less oxygen available for diffusion into the egg. For example, crowding of eggs in rainbow trout leads to more stagnant water around the eggs as development progresses, creating an environment with lower oxygen availability (Dhiyebi *et al.*, 2013).

There was no significant difference observed in the percent of fertile eggs laid per clutch between CO_2 treatments. However, the second clutch from two different adult pairs within the elevated CO_2 treatment were extreme outliers, with only one to two fertile eggs present. All other clutches from both treatments had between 87 and 100 percent fertile eggs. It is possible that fertility could tend to decline in later egg clutches under elevated CO_2, but no conclusions can be drawn from the current results. It would be worth reinvestigating with a larger sample size to determine if there is a possible effect of elevated CO_2 on fertility rate over multiple clutches. There was also no difference between CO_2 treatments in the percentage of animals that successfully hatched or the mean time to hatch.

This study has demonstrated a number of effects of elevated CO_2 on the reproduction, development, and spawning behaviours of *I. pygmaeus*. While the increased swelling of eggs during late stage development at elevated CO_2 could be an adaptive response to elevated CO_2, reduced clutch size and smaller hatching size could have an adverse effect on squid populations. Many of the effects observed here, such as smaller clutch size and reduced embryo size, were apparent immediately after spawning. Furthermore, the differences in the size of the embryos between CO_2 treatments became less throughout development. I expect

that these size differences between CO_2 treatments at later embryonic stages are due to initial size differences rather than as a result of egg exposure. This links all negative effects seen in this experiment to parental exposure, leading me to conclude that parental exposure to elevated CO_2 has significant negative effects on adult energy budgets. Cephalopods occupy an important central location within marine food webs, as both predator and prey (Hanlon & Messenger, 2018). Therefore, changes to their population structure as a result of the reproductive effects of elevated CO_2 could have consequences for other species that prey upon them. The small size of *I. pygmaeus* paralarvae, compared to other squid, may place them in an especially vulnerable position due potential predation. However, pygmy squid also have short generation times, living for just 90 days on average. Therefore, they may have a much greater capacity to adapt to higher CO_2 levels in the future than longer lived marine species. Cephalopods have persisted through many environmental changes during their evolutionary history (Boyle & Rodhouse, 2005) and they may be better able to adjust to rapid environmental change than many other marine species. Future studies should investigate the adaptive capacity of *I. pygmeaus*, and other cephalopod species, to the rapid pace of change in ocean chemistry being caused by anthropogenic CO_2 emissions.

Chapter 4: Predatory strategies and behaviours in cephalopods are altered by elevated carbon dioxide

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4.1 – Summary

There is increasing evidence that projected near-future carbon dioxide (CO_2) levels can alter predator avoidance behaviour in marine invertebrates, yet little is known about the possible effects on predatory behaviours. Here I tested the effects of elevated CO_2 on the predatory behaviours of two ecologically distinct cephalopod species, the pygmy squid, *Idiosepius pygmaeus*, and the bigfin reef squid, *Sepioteuthis lessoniana*. Both species exhibited an increased latency to attack and altered body-pattern choice during the attack sequence at elevated CO_2. *I. pygmaeus* also exhibited a 20% decrease in predation rate, an increased striking distance, and reduced preference for attacking the posterior end of prey at elevated CO_2. Elevated CO_2 increased activity levels of *S. lessoniana* comparable to those previously shown in *I. pygmaeus*, which could adversely affect their energy budget and increase their potential to be preyed upon. The effects of elevated CO_2 on predatory behaviours, predation strategies and activity levels of cephalopods reported here could have far-reaching consequences in marine ecosystems due to the ecological importance of cephalopods in the marine food web.

4.2 – Introduction

Atmospheric carbon dioxide (CO_2) concentrations have increased by more than 40%, from 280 ppm before the industrial revolution to over 400 ppm in 2016 (Dlugokencky & Tans, 2016), and are now higher than any time in the past 800,000 years (Lüthi *et al.*, 2008). Climate models project that atmospheric CO_2 will exceed 900 ppm by the end of this century on the current emissions trajectory (Collins *et al.*, 2013) with a rate of increase at least an order of magnitude faster than at any time in the past million years (Doney & Schimel, 2007). The partial pressure of CO_2 (pCO_2) in the surface ocean is in approximate gas equilibrium with atmospheric CO_2, and therefore is rising at the same rate as atmospheric CO_2 (Doney, 2010). Furthermore, seasonal fluctuations in ocean pCO_2 are projected to be amplified in the future due to the increased Revelle (buffer) factor of acidified seawater (McNeil & Sasse, 2016). The increase in average pCO_2 combined with amplification of seasonal cycles of pCO_2 suggests that marine organisms will experience CO_2 levels greater than 1,000 µatm for many months each year by around 2080 (McNeil & Sasse, 2016). These relatively rapid changes to ocean chemistry could potentially affect many marine species and have substantial impacts on marine ecosystems (Hoegh-Guldberg *et al.*, 2007; Sabine & Feely, 2007; Fabry *et al.*, 2008; Doney *et al.*, 2009).

Elevated CO_2 levels can have many adverse effects on marine animal physiology (Pörtner *et al.*, 2004), life history traits (Kroeker *et al.*, 2013), and behaviour (Nagelkerken & Munday, 2016). Much of the research into the behavioural effects of elevated CO_2 has focused on fish (Heuer & Grosell, 2014), showing multiple adverse effects to sensory systems (Munday *et al.*, 2009b, 2010; Simpson *et al.*, 2011) and influencing ecologically important behaviours, such as predator-prey interactions (Cripps *et al.*, 2011; Ferrari *et al.*, 2011a; Allan *et al.*, 2013;

Ferrari *et al.*, 2015; Pistevos *et al.*, 2015, 2016). New studies have also demonstrated the behavioural impacts of elevated CO_2 on marine molluscs. For example, elevated CO_2 impairs predator avoidance behaviour in intertidal snails (Manríquez *et al.*, 2013, 2014; Jellison *et al.*, 2016). In the jumping conch snail, elevated CO_2 affects predator-escape behaviour, reducing the proportion of snails that jump from a predator, and increasing the latency to jump, as well as altering the escape trajectories of snails that do jump to escape predation (Watson *et al.*, 2014). More recently elevated CO_2 has been shown to cause a reduction in the predation rates of the predatory marbled cone snail; 60% of control snails successfully captured and consumed their prey compared with 10% at elevated CO_2, despite a threefold increase in activity levels at elevated CO_2 (Watson *et al.*, 2017). To date, studies on the potential effects of elevated CO_2 on more advanced and active molluscs, such as cephalopods, have largely focused on physiological traits.

The physiological effects of elevated CO_2 on cephalopods have been investigated in a range of species. Elevated CO_2 has been shown to cause metabolic suppression in squids (Rosa and Seibel, 2008; Hu *et al.*, 2014), however, Gutowska *et al.* (2008) found no effect of elevated CO_2 on the metabolic rates of a cuttlefish species. There are also adverse effects of elevated CO_2 on cephalopod growth and development (Hu *et al.*, 2011a; Kaplan *et al.*, 2013; Sigwart *et al.*, 2016). However, the acid-base regulation capabilities of cephalopods are very advanced, which could give them an advantage over other species in future acidified oceans (Gutowska *et al.*, 2010; Hu *et al.*, 2013, 2014). Only two studies to date have investigated the potential effects of elevated CO_2 on cephalopod behaviour. In the two-toned pygmy squid, *Idiosepius pygmaeus,* elevated CO_2 increased the proportion of squid that were active and resulted in a near three-fold increase in activity levels among non-resting individuals compared to control

CO_2 conditions (Spady *et al.*, 2014). Elevated CO_2 also altered defensive responses to a visual predator cue. Squid were more likely to ink and jet escape, as well as altered body pattern choice at elevated CO_2, although their time to react to the cue and ability to escape was not inhibited, indicating that their decision-making process was altered. In contrast to the increase in activity demonstrated by Spady *et al.* (2014), a recent study shows elevated CO_2 causes a decrease in activity levels and reduction in horizontal velocity in paralarval squid, *Doryteuthis pealeii* (Zakroff *et al.*, 2017). These results raise the question of whether other behaviours and decision-making processes in cephalopods, such as predatory behaviours, will also be altered by rising CO_2.

Cephalopods comprise approximately 800 extant species found throughout the world's oceans in a diverse range of ecosystems (Coll *et al.*, 2013). They prey on a wide range of marine organisms (e.g., fishes, crustaceans, bivalves, other cephalopod species, and small planktonic species) and are also preyed upon by many predator species, including cetaceans, pinnipeds, marine birds, turtles, and a wide range of teleost fishes and sharks (Hanlon & Messenger, 2018). The high feeding rates and abundance of squids and other cephalopods have the potential to control the recruitment of many commercially and ecologically important fishes (Hunsicker & Essington, 2008). Consequently, any changes to the predator-prey dynamics of cephalopods could have ramifications for ecosystem structure.

In this study, I tested the effects of projected near-future CO_2 levels on the predatory behaviours of two tropical cephalopod species from separate taxonomic orders that occupy largely different ecological niches. The two-toned pygmy squid, *I. pygmaeus*, was used to compare any potential predation effects of elevated CO_2 with known effects on their

antipredator behaviour (Spady *et al.*, 2014). Pygmy squid are found in the Indo-Pacific from the South China Sea to northern Australia, inhabiting shallow coastal waters (Semmens *et al.*, 1995; Barratt & Allcock, 2013). They have a life span of roughly 90 days and due to their habitat and small size (mantle length up to 21 mm), are likely to be important prey for juveniles of some coastal fishes and predators of small pelagic invertebrates (Moynihan, 1983; Hanlon & Messenger, 2018). Here, I tested the effects of current-day control (438 µatm) and two projected near-future CO_2 levels (737 and 934 µatm) on the predatory interactions of pygmy squid with a common prey item, the glass shrimp, *Acetes sibogae australis* (Jackson, 1992). The CO_2 treatments were selected to match projected CO_2 levels in the atmosphere and ocean by the end of this century for a moderate and high emissions trajectory based on representative concentration pathways (RCP) 6.0 and 8.5 respectively (Collins *et al.*, 2013).

The second species used in this study, the bigfin reef squid (*Sepioteuthis lessoniana*), is a much larger species (mantle length to 36 cm) and occupies a wider range of habitats. Bigfin reef squid are found in waters of up to 100 m deep in tropical seas and move into coral reef habitats at night to feed on a variety of fish and crustaceans (Norman, 2003). I exposed bigfin reef squid to current-day control (435 µatm) and projected future CO_2 levels (935 µatm) following RCP8.5 (Collins *et al.*, 2013), and tested their predatory interactions with a common reef fish, the spiny chromis damselfish, *Acanthochromis polyacanthus*. Additionally, the activity levels of bigfin reef squid were also compared between CO_2 treatments to determine if elevated CO_2 increases activity as observed in pygmy squid (Spady *et al.*, 2014), or decreases activity, as seen in paralarval *D. pealeii* (Zakroff *et al.* 2017). It is important to note that while commonly called 'pygmy squid', *I. pygmaeus* is in the order Idiosepiida and is more closely related to cuttlefish (Sepiida) than to a true squid, such as *S. lessoniana*, of the order Teuthida.

I predicted that elevated CO_2 may alter predatory strategies during an interaction with prey for both orders of cephalopod investigated in this study, but that their physical ability to attack and capture their prey would not be diminished.

4.3 – Methods

CO_2 treatment systems

Experiments were conducted in 8,000 L recirculating seawater systems at James Cook University's research aquarium in Townsville, Australia. A pH control system (AT Control, Aqua Medic, Germany) dosed CO_2 into 3,000 L sumps to achieve the desired pH level for each CO_2 treatment. pH_{NBS} was measured daily (Seven2Go Pro, Mettler Toledo, Switzerland) and dosing set points were adjusted as necessary to maintain the target pCO_2 in each treatment. Equilibrated seawater from each system was delivered at a rate of 1.5 L min^{-1} to aquaria containing squid. Temperature was measured daily in each tank (Comark C26, Norfolk, UK).

Water samples were taken weekly to determine pH on the total scale (pH_T) by spectrophotometry (UVmini-1240, Shimadzu, Suzhou Instruments Co. Ltd., Kyoto, Japan) using m-cresol purple as an indicator dye (Dickson & Millero, 1987; Dickson *et al.*, 2007). Comparison of pH_{NBS} and pH_T were used to estimate daily pH_T values. Total alkalinity was estimated weekly by Gran Titration (888 Titrando Metrohm AG, Switzerland) ensuring titration calibrations remained within one percent of certified reference material from Prof. A.G. Dickson (Scripps Institution of Oceanography, batch #135). Salinity was measured weekly using a conductivity sensor (HQ15d, Hach, Loveland, CO, USA). Carbonate chemistry parameters (**Table 4.1**) were calculated in CO2SYS (Pierrot *et al.*, 2006) using the constants K1, K2 from Mehrbach *et al*. (1973) refit by Dickson and Millero (1987) and Dickson *et al.*

(2007) for $KHSO_4$. Temperature, salinity, and control pH were set to replicate natural conditions of the waters in which animals were captured.

Table 4.1 – Seawater carbonate chemistry – The temperature, salinity, $pH_{(T)}$, total alkalinity, and pCO_2 for each CO_2 treatment level and species. Values are means ± SD.

Species	Treatment	Temperature (˚C)	Salinity	$pH_{(T)}$	Total alkalinity (µmol kg^{-1} SW)	pCO_2 (µatm)
Idiosepius pygmaeus	Control	29.3 (±0.4)	35.2 (±1.7)	8.02 (±0.09)	2368 (±299)	438 (±80)
	Moderate-CO_2	29.3 (±0.3)	35.2 (±1.4)	7.84 (±0.05)	2392 (±209)	737 (±107)
	High-CO_2	29.0 (±0.9)	36.0 (±1.2)	7.74 (±0.05)	2348 (±319)	934 (±152)
Sepioteuthis lessoniana	Control	28.5 (±0.4)	36.1 (±0.5)	7.99 (±0.05)	2194 (±56)	435 (±62)
	High-CO_2	28.6 (±0.3)	36.2 (±0.5)	7.71 (±0.03)	2204 (±109)	935 (±93)

I. pygmaeus collection and experimental trials

Two-toned pygmy squid were collected between January-March 2016 from Cleveland Bay in Townsville, Queensland, Australia (19˚24'S, 146˚82'E). A total of 150 were captured in the early morning with a 500 µm-mesh triangular frame dip net and transported immediately to James Cook University, Townsville. Squid were kept at control conditions for 24-hours in separate holding tanks before being transferred to their CO_2 treatment tanks for five days. CO_2 levels were set at: 1) a current-day control (438 µatm), 2) a moderate end-of-century projection RCP6.0 (moderate CO_2, 737 µatm) and 3) an upper end-of-century projection RCP8.5 (high CO_2, 934 µatm) (Collins *et al.*, 2013). During CO_2 treatment, squid were housed in round tanks (47Ø x 51H cm) filled with seawater to 67-litres. Each tank contained two to five individuals, and there were 20 tanks at each CO_2 level (pygmy squid sample size, control: n=47, moderate: n=49, high: n=47). Slightly unequal sample sizes were due to low levels of natural mortality during the treatment period (**Table S3**). Animals were provided with PVC pipe structures in the tanks as shelter. Pygmy squid quickly adjust to captivity

(Moltschaniwskyj *et al.*, 2007); individuals in the current experiment readily ate and were commonly observed copulating and laying eggs in all CO_2 treatments. Animals were fed glass shrimp (*Acetes sibogae australis*) *ad libitum* daily in the morning. Shrimp remained in the tank throughout the day and squid were observed regularly feeding throughout the day. Glass shrimp were captured in the same location as the pygmy squid and kept at control conditions. Food was withheld on the day of behavioural trials. A treatment period of five days was chosen due to the short life span of *I. pygmaeus* and because previous research on this species has demonstrated behavioural effects of elevated CO_2 are evident after this exposure period (Spady *et al.*, 2014). Furthermore, the behavioural effects resulting from short-term exposure to elevated CO_2 (four days) in fish is not ameliorated following longer exposures of weeks to months (Munday *et al.*, 2013, 2014; Welch *et al*., 2014).

Predator-prey interactions between pygmy squid and glass shrimp were tested after five days in CO_2 treatment. Individual squid were carefully transferred from their holding tank to a clear plastic arena (34L x 20W x 18H cm) containing 11 litres of seawater from their CO_2 treatment. Squid were left to habituate in the arena for 3 min before the prey was introduced. A single prey glass shrimp was held in a 1 mL transfer pipette, 5 cm above the arena. After the three-minute habituation period, the pipette bulb was squeezed (out of view of the squid) introducing the glass shrimp into the arena. Interactions were filmed from directly above the arena, 25 cm from the water surface, with a digital camera (Canon PowerShot G16) at 30fps. Filming continued until either the squid had successfully captured the prey, or for a total of 10 min if the prey was not captured. The total length (TL) of glass shrimp was recorded before the start of each trial and squid mantle length (ML) was measured at the conclusion of each trial.

S. lessoniana collection and experimental trials

Bigfin reef squid were collected during May and June 2016 from the Townsville breakwater, Queensland, Australia (19˚24'S, 146˚82'E). A total of 42 were captured at night with a 2.5 cm mesh, round frame dip net and transported immediately to James Cook University, Townsville. Squid were kept individually in round tanks (47Ø x 51H cm) filled to 67-litres, with a continuous flow of seawater. Individuals were kept at control conditions for 21 days before being moved to their CO_2 treatment tanks where they remained for 28 days before the behavioural trials (bigfin reef squid sample size, control: n=19, high: n=21). Slightly unequal sample sizes were due to low levels of natural mortality during the treatment period (**Table S3**). Two CO_2 levels were used: 1) a current-day control (435 µatm) and 2) an upper end-of-century projection RCP8.5 (high CO_2, 935 µatm). A moderate CO_2 treatment was not used in this experiment as the number of bigfin reef squid captured was insufficient for appropriate replication in three treatment levels. Squid were fed a variety of live food, including locally caught estuary glassfish (*Ambassis marianus*), juvenile flathead grey mullet (*Mugil cephalus*), as well as spiny chromis damselfish (*Acanthochromis polyacanthus*), two times daily. Feeder fish were kept at control CO_2 conditions prior to feeding. Spiny chromis damselfish were used as prey fish in the experimental trials. Prey fish were maintained in both control or high CO_2 conditions. Fish from each holding condition were then randomly assigned to the control or high CO_2 treatment in the predator-prey trials to account for any potential effect of a change in treatment water on prey fish behaviour when placed into the testing arena. A treatment period of 28 days was chosen as previous experiments on the species have shown that acid-base regulation had fully restored extracellular pH within 20 hours, yet there were still physiological effects of elevated CO_2 after a treatment period of seven days (Hu *et al*., 2014).

The treatment period of 28 days accounts for more than 15% of the total average lifespan of this species in the wild.

Activity levels of bigfin reef squid, as well as the predator-prey interactions between squid and the spiny chromis were tested after 28 days in CO_2 treatment. Individual squid were carefully transferred from their holding tanks to a white plastic arena (68L x 50W x 43H cm) containing 75 L of flow-through water from their treatment. A black plastic sheet separated the arena from the viewer and cameras. Cameras were set up to view a mirror through a hole in the sheet, which was angled down to provide a bird's eye view of the arena; a halogen lamp (150 watts) was on the ground illuminating the ceiling, providing enough light for the arena. Squid were left to habituate for 45 min. After this habituation period, squid activity was filmed (Canon PowerShot G16) for 3 min. Following this, after a total of 48 min, a second video camera (Casio Exilm HS EX-ZR2000) began recording at 120fps and a single damselfish was introduced to the arena. Interactions were filmed until either the squid had successfully captured the prey, or for 25 min if the prey was not captured. The TL of damselfish was recorded 45 min before the start of each trial and squid ML was measured at the conclusion of each trial.

Behavioural analysis

Prey interaction videos from both experiments were viewed with the observer blinded to the treatment. The following information was extracted from the videos with Quicktime 7 Pro video software (Apple Inc., Quicktime 7 Pro v.7.6.6):

- proportion of animals that attacked prey
- latency to attack – from when prey is introduced to when attack pose begins

- time holding attack pose – squid aligns anteroposteriorly with prey and streamlines mantle and arms, concluded when strike with tentacles is initiated
- striking distance – the distance from the tentacles to the prey when arms are splayed open to expose striking tentacles
- attack direction – the angle of the squid in relation to the prey at time of attack in which the posterior end of the of the prey is 0˚ and the anterior end is 180˚; Attacks were defined as 'posterior' if less than 45˚, 'lateral' if between 45˚ and 135˚, and 'anterior' if greater than 135˚
- body pattern – choice of body pattern during the attack pose, categorised as 'dark mottle' or a more transparent 'uniform blanch' (Hanlon and Messenger, 1988)
- proportion of animals that captured prey on first strike attempt

For bigfin reef squid, LoliTrack software (Loligo Systems v4.2) was used to extract the total distance moved, proportion of time the squid was active, and average speed from videos.

Statistical analysis

Statistical analyses were performed using R statistical software (R). For all statistics with pygmy squid, tank was included as a random factor and the fixed factors were squid CO_2 treatment, squid sex, and the size ratio between predator and prey. For bigfin reef squid, fixed factors for behaviours in the predatory experiment were squid CO_2 treatment, squid sex, the size ratio between predator and prey, and CO_2 treatment of the prey fish. Generalised linear mixed models (GLMM) and generalised linear models (GLM) with quasipoisson distributions were used to compare latency to attack, time holding attack pose, and striking distance in pygmy squid and bigfin reef squid, respectively. GLMMs (pygmy squid) and GLMs

(bigfin reef squid) with binomial logistic regressions were used to compare the proportion of animals that attacked the prey, proportion of animals that successfully captured the prey, and body pattern choice. Pygmy squid attack direction was analysed using a GLMM with a binomial logistic regression, comparing the preferred "posterior" attacks to "non-posterior" attacks in order to include tank as a random factor. Bigfin reef squid attack direction was analysed using a GLM with a multinomial regression. For the bigfin reef squid activity level experiment, GLMs with quasipoisson distributions were used with squid CO_2 treatment, squid sex, and squid mantle length as fixed factors. CO_2 treatments were treated as ordinal data by transforming pCO_2 into a numeric value as a multiplier of the control value for each experiment, so that, for example, 438, 737, and 934 µatm CO_2 became 1, 1.681, and 2.113 respectively.

4.4 – Results

Predatory behaviours – *I. pygmaeus*

For pygmy squid, there was an approximately 20% decrease in the proportion of individuals that attacked the prey at elevated CO_2 compared with the control (GLMM, χ^2=4.441, p=0.035) (**Fig. 4.1a**). During the 10-minute trial, 85% of squid from the control treatment made a predatory strike at the prey, compared to 63% and 67% from the moderate and high CO_2 treatments, respectively.

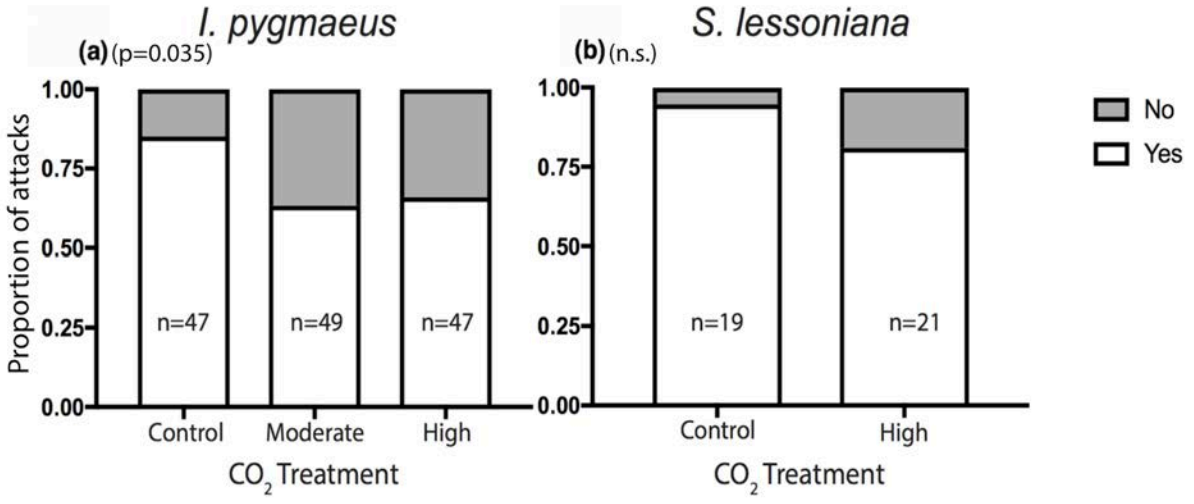

Figure 4.1 – Predatory attack occurrence – Proportion of individuals that made a predatory attack on their prey item at each CO_2 treatment level for (a) *I. pygmaeus* and (b) *S. lessoniana*; white bars indicate an attack, grey bars indicate no attack was made. The p-values are displayed on graphs containing a significant difference among CO_2 treatments. A non-significant relationship is indicated by 'n.s.'.

In squid that did attack the prey, elevated CO_2 increased the time to initiate an attack by greater than one minute (GLMM, χ^2=7.639, p=0.006) (**Fig. 4.2a**). Control squid had a mean latency to attack of 91 seconds, whereas the moderate and high CO_2 squid had a latency to attack of 171 and 185 seconds, respectively. Furthermore, the mean time holding the attack pose (from initiation to striking) increased by 45% from the control to high CO_2 treatment, however the effect of CO_2 was not statistically significant (GLMM, χ^2=2.899, p=0.089) (**Fig. 4.2c**). The distance from which squid made their attacking lunge at the prey was significantly different among CO_2 treatments (GLMM, χ^2=6.529, p=0.011) (**Fig. 4.2e**). Squid at control CO_2 had a mean attack distance of 19.7 mm, compared to the moderate and high CO_2 squid, which attacked from 30.8 mm and 29.3 mm respectively.

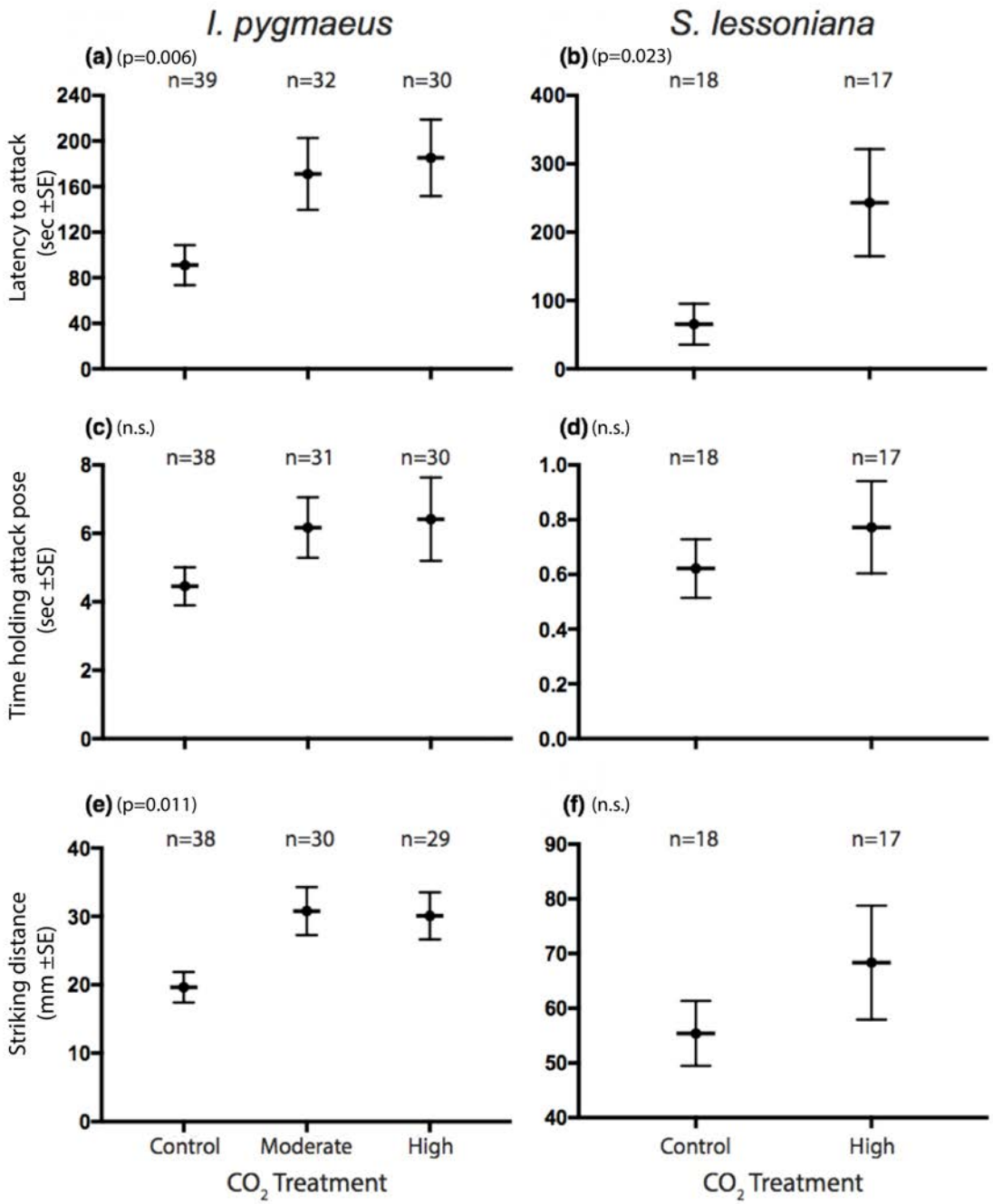

Figure 4.2 – Latency to attack and strike distance – Mean time (sec ±SE) to initiate a predatory attack sequence after the introduction of prey in both (a) *I. pygmaeus* and (b) *S. lessoniana*. Mean time (sec ±SE) holding the attacking pose before striking the prey in (c) *I. pygmaeus* and (d) *S. lessoniana*. Mean distance (mm ±SE) from prey when striking tentacles are utilised in both (e) *I. pygmaeus* and (f) *S. lessoniana*. The p-values are displayed on graphs containing a significant difference among CO_2 treatments. A non-significant relationship is indicated by 'n.s.'.

There was a significant difference among CO_2 treatments in the body pattern choice of squid during their attack (GLMM, χ^2=5.571, p=0.018) (**Fig. 4.3a**). The uniform blanch display was used in nearly 95% of attacks in control CO_2 and 90% in the moderate CO_2 treatment, but decreased to 70% in the high CO_2 treatment. Squid that did not exhibit the uniform blanch display, instead expressed a dark mottle display. The attack direction of the squid was also significantly different among CO_2 treatments (GLMM, χ^2=4.260, p=0.039) (**Fig. 4.3c**). The proportion of attacks from the posterior end of the prey decreased from 89% in control CO_2 to 67% in the high CO_2 treatment and lateral attacks increased from 3% in control to 30% in high CO_2. There was no difference in the proportion of squid that successfully captured the prey on their first strike attempt (GLMM, χ^2=0.039, p=0.843), which was between 83% and 85% in all three treatments. Squid sex and the size ratio between predator and prey had no effect on pygmy squid predatory behaviours (**Table S4**).

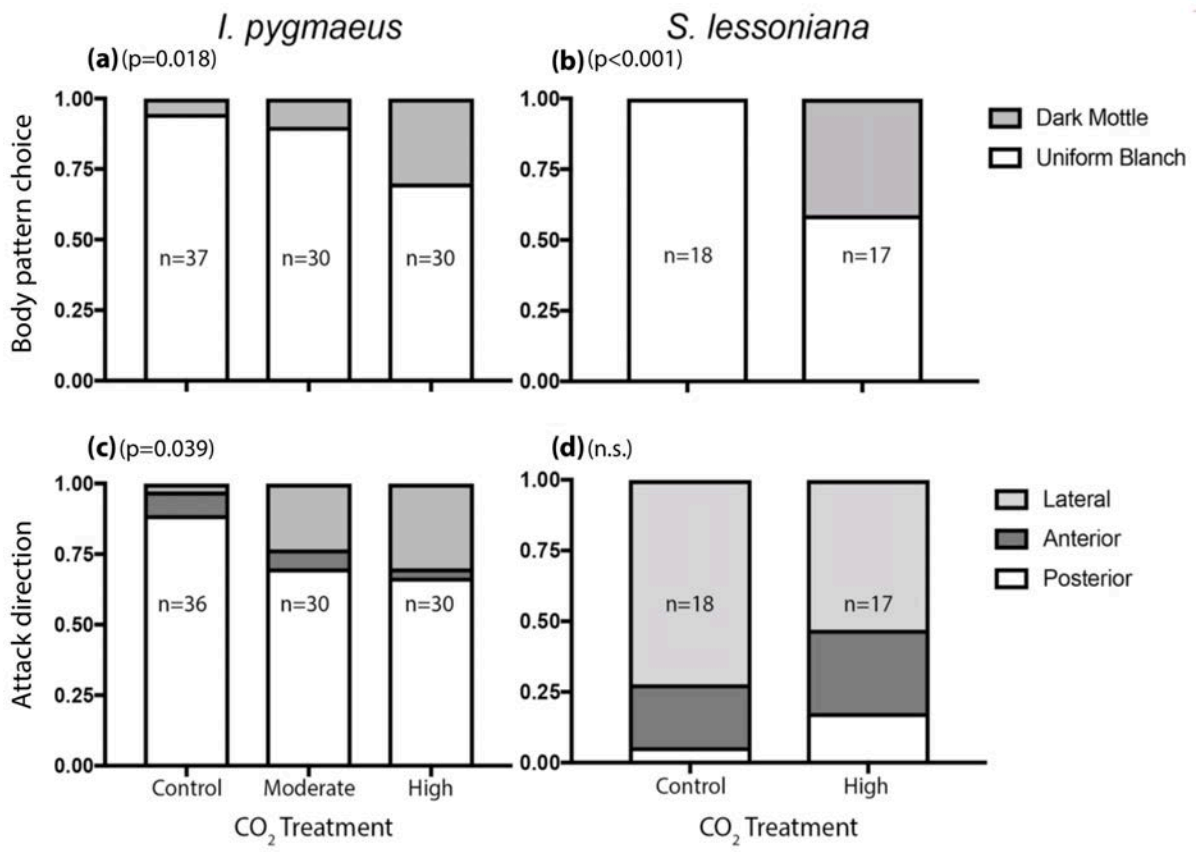

Figure 4.3 – Body pattern and attack direction – Proportion of individuals that chose each body pattern during the predatory attack sequence at each CO_2 treatment level in (a) *I. pygmaeus* and (b) *S. lessoniana*. White bars indicate animals choosing a uniform blanch body display during the attack, grey bars indicate animals choosing a dark mottle display. The direction from which the predator made the predatory strike on the prey in (c) *I. pygmaeus* and (d) *S. lessoniana*. White bars indicate an attack on the posterior end of the animal, dark grey bars indicate an attack on the anterior end, and light grey bars indicate a lateral attack. The p-values are displayed on graphs containing a significant difference among CO_2 treatments. A non-significant relationship is indicated by 'n.s.'.

Predatory behaviours – *S. lessoniana*

For the bigfin reef squid, there was no effect of elevated CO_2 on the proportion of individuals that attacked the prey (GLM, χ^2=1.809, p=0.179) (**Fig. 4.1b**). Control squid made a predatory strike at the prey in 95% of the trials, while squid kept at high CO_2 attacked in 81% of the trials. However, in squid that did attack the prey, there was a more than 250% increase in the latency to attack seen in squid held at high CO_2 compared with control squid (GLM, χ^2=5.167, p=0.023) (**Fig. 4.2b**). The mean time to initiate an attack was 66 seconds for control squid,

compared to 243 seconds in the high CO_2 squid. There was no effect of CO_2 on the time holding the attack pose (GLM, χ^2=0.952, p=0.329) (**Fig. 4.2d**) or in the distance from which bigfin reef squid attacked the prey (GLM, χ^2=1.319, p=0.251) (**Fig. 4.2f**).

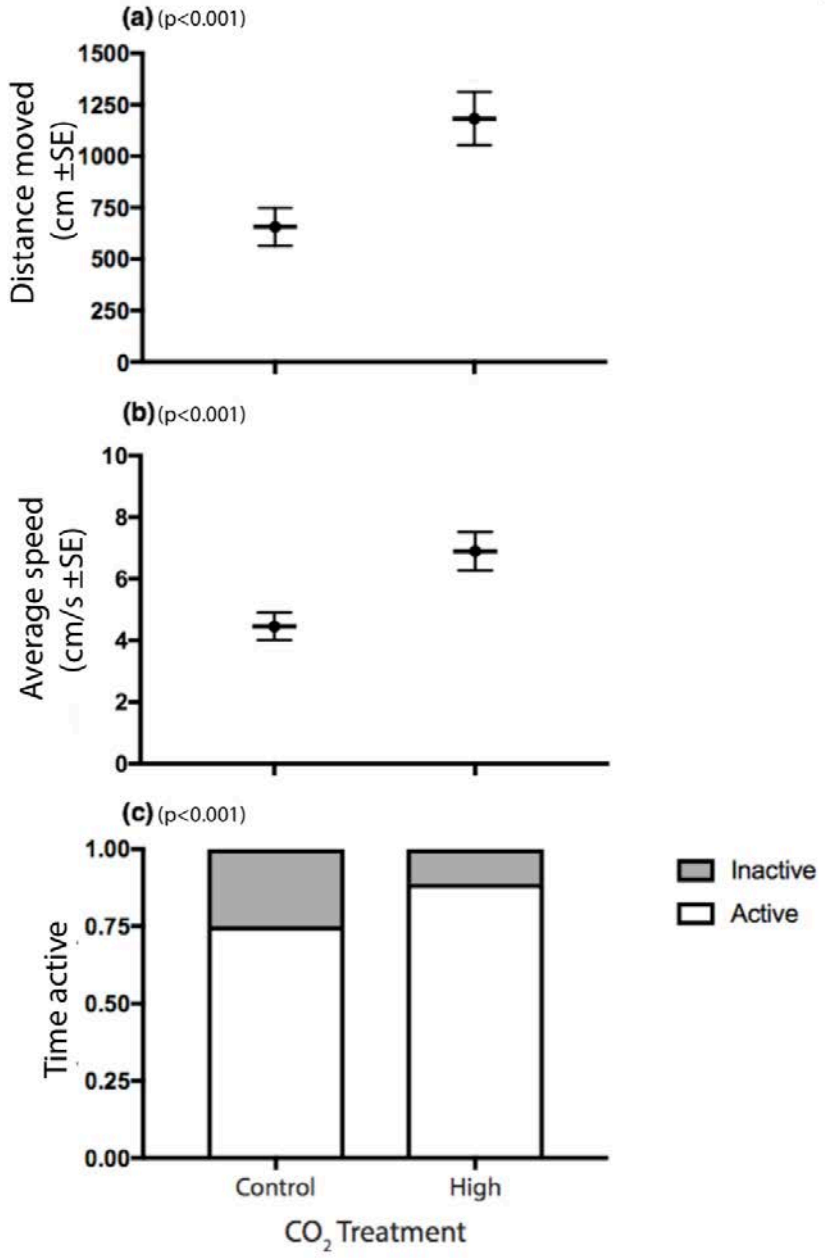

Figure 4.4 – Activity levels – The activity levels of *S. lessoniana* during a 3-minute trial at both control (n=18) and elevated CO_2 (n=20). Activity was quantified as: (a) mean distance moved (cm ±SE), (b) mean speed (cm/sec ±SE), and (c) proportion of time animals spent being active (white) compared to being inactive (grey). The p-values are displayed on graphs containing a significant difference among CO_2 treatments.

The body pattern choice of squid during attack was significantly different between treatments (GLM, χ^2=12.123, p<0.001) (**Fig. 4.3b**) with 100% of individuals from the control treatment exhibiting a uniform blanch display, whereas 41% of squid from the high CO_2 treatment instead chose to use a dark mottle display during the attack sequence. There was no significant difference in the direction of attack between treatments (GLM, χ^2=2.059, p=0.357) (**Fig. 4.3d**). The proportion of individuals that successfully captured their prey on the first strike attempt did not differ between CO_2 treatments (GLM, χ^2=1.499, p=0.221). The sex of bigfin reef squid had an effect only on capture success, with females being more likely to capture prey at both CO_2 treatments. The CO_2 treatment of prey and the size ratio between predator and prey had no effect on bigfin reef squid predatory traits (**Table S4**).

Activity levels – *S. lessoniana*

The total distance moved by squid during the three-minute activity trials nearly doubled from a mean of 656 cm at control CO_2 to 1,182 cm at high CO_2 (GLM, χ^2=20.647, p<0.001) (**Fig. 4.4a**). The mean speed of squid between treatments significantly increased from 4.46 cm/s at control to 6.89 cm/s at high CO_2 (GLM, χ^2=18.536, p<0.001) (**Fig. 4.4b**). Lastly, in comparing the amount of time the squid remained active throughout the trial, there was a 13% increase in the high CO_2 treatment compared to control (GLM, χ^2=14.414, p<0.001) (**Fig. 4.4c**). Control squid were active for a mean of 75% of the trial, while squid from the high CO_2 treatment were active for over 88% of the trial. The sex of bigfin reef squid had no effect on activity level traits. There was, however, a significant positive correlation between squid mantle length and all activity level traits (**Table S4**).

4.5 – Discussion

I found that exposure to projected near-future CO_2 levels affected predatory behaviours of two ecologically divergent species of tropical cephalopod - the two-toned pygmy squid and bigfin reef squid. Elevated CO_2 caused a significant reduction in the proportion of pygmy squid that attacked their prey, and although not statistically significant, a similar proportional reduction in attacks was observed in the bigfin reef squid. For the animals that attacked the prey, the time taken to initiate attack increased significantly at elevated CO_2 in both species. At elevated CO_2, the striking distance of pygmy squid also significantly increased. Both species showed alterations in their choice of body pattern during the attack sequence at elevated CO_2; while there was a clear preference for the uniform blanch body display in controls, there was a significant increase in the dark mottle pattern in squid exposed to high CO_2. The attack direction of pygmy squid was also altered by elevated CO_2: the preference for attacks on the posterior end of the prey decreased while lateral attacks increased. Despite these changes in behaviour during the predatory sequence, there were no significant differences in the first strike capture success of either species. Additionally, activity levels of the bigfin reef squid changed at elevated CO_2, with increased total distance moved, average speed, and time active.

The proportion of pygmy squid that attacked their prey was about 20% lower at high CO_2 compared to at control. This is comparable to the decrease in predation rates of fish (Allan *et al.*, 2013), copepods (Cripps *et al.*, 2016), and a gastropod mollusc (Watson *et al.*, 2014) observed when kept at similar CO_2 levels. Cephalopods have an extremely rapid growth rate and are voracious carnivores to keep up with their fast metabolisms (Hanlon & Messenger, 2018). A reduction in the desire to attack prey could conflict with the high energy demands

of squids required for their fast metabolic rates. A reduction in predation rates may however be a consequence of changes in their metabolism resulting from elevated CO_2. However, the aerobic scope of both species studied here are not affected by similar elevated CO_2 levels (**Chapter 2**), indicating no alterations to their metabolic rates. Regardless of the explanation, cephalopods are important predators in the marine food web, and changes to their feeding rates could have significant impacts on ecosystem structure.

In both species, for individuals that did attack their prey, I found multiple changes to their predatory strategies as a result of elevated CO_2. Time to initiate an attack increased by over one minute in pygmy squid, and by over three minutes in bigfin reef squid at elevated CO_2. An increase in time to initiate attack in the wild could potentially result in squids missing their 'window of opportunity' for successful capture of some prey species. Furthermore, the striking distance chosen by pygmy squid was greater than one average body length more at elevated CO_2. This could provide prey more time to recognise an oncoming attack, and prey species other than those tested here may have a greater chance of escape in the wild. If projected near-future CO_2 levels result in squids missing their opportunity to attack, or alter their behaviour in a way that gives their prey greater opportunity to escape, there could be a reduction in prey capture by squids. This again could be costly to their high-energy demands for growth and basic metabolic processes (Hanlon & Messenger, 2018).

The shift in the preference of attack direction of pygmy squid from posterior attacks to lateral attacks at elevated CO_2 could also give some prey species a greater opportunity of escape in the wild, as the squid would be in the prey's field of vision during lateral attacks. While some cephalopod species attack prey fish and prawns from any direction (Neill & Cullen, 1974),

here pygmy squid show a clear preference for posterior attacks (89%) in current-day control conditions. By contrast, there was no change in the direction of attack made by bigfin reef squid, which showed a preference for lateral attacks at both treatment levels. The preference for posterior attacks in pygmy squid could be due to the difference in size ratios of predator and prey during predator-prey interactions. The mantle length of pygmy squid was on average less than 60% of the average total length of the prey glass shrimp, whereas in the trials with bigfin reef squid, the mantle length of squid was on average greater than 200% larger than the average total length of prey damselfish. Because the prey used in pygmy squid trials were much larger in relation to the squid, a posterior attack angle may have been more critical to successfully capture and avoid injury. Future studies could consider the predator to prey size ratio on the attack angles of cephalopods.

The choice of body pattern display in both squid species during the attack sequence was also altered by elevated CO_2. This was especially evident in bigfin reef squid, where 100% of squid at control chose the uniform blanch body display and this decreased by nearly half in exchange for the dark mottle display at high CO_2. The change in body patterns in this study is notable because utilising the dark mottle display at elevated CO_2 caused squid to have a much greater contrast to the white background of the test arenas. When squid attacked with the uniform blanch body pattern, their transparent appearance much more easily blended in with the background shade. This indicates that their predatory strategy during the attack may be altered in a potentially disadvantageous way by making them more visible to their prey during the attack. In pygmy squid, Spady *et al*. (2014) found that body pattern choice during predator avoidance is also altered by elevated CO_2. These findings suggest that the complex use of body colouration by cephalopods for crypsis and threat signals during predator-prey

interactions may be impaired by elevated CO_2. Cephalopods use fast neural control when regulating the muscles related to their chromatophore organs (responsible for changing their body colours and patterns) (Hanlon, 2007; Hanlon *et al.*, 2009), and they are capable of deciding whether to be cryptic or conspicuous depending on their situation and external factors (Adamo *et al.*, 2006; Zylinski *et al.*, 2011). This complex network of millions of neurons responsible for altering body patterns in cephalopods contain "all of the 'classic' neurotransmitters", including γ-aminobutyric acid (GABA) which are distributed throughout all neural lobes controlling chromatophore function (Messenger, 2001). $GABA_A$ receptors are important in this context as they appear to be responsible for the behavioural changes in other molluscs (Watson *et al.*, 2014) and fishes (Nilsson *et al.*, 2012; Hamilton *et al.*, 2014; Lai *et al*., 2015; Heuer *et al*., 2016) resulting from elevated CO_2. Recent molecular analyses also demonstrate that substantial changes to GABA pathways are a feature of high CO_2 exposure in other invertebrates (Moya *et al.* 2016) and fish (Schunter *et al*. 2018), further supporting a role in behavioural changes. Cephalopods exhibit some of the most remarkable and flexible cryptic capabilities in the animal kingdom by mimicry of the surrounding environment (Hanlon & Messenger, 2018). Changes in the ability of cephalopods to effectively utilise their cryptic capabilities would remove one of their most unique and important advantages over both predators and prey, potentially causing higher levels of mortality and a shift in their ecological position.

In both species, there were marked changes in the predatory behaviours, yet individuals that chose to attack the prey showed no difference among CO_2 treatments in the proportion of successful captures on the first attempt. This suggests that squid may retain the physical capabilities needed to capture the prey at elevated CO_2, but their decision-making processes

regarding predatory strategies are altered at elevated CO_2. Similarly, Spady *et al*. (2014) found no inhibition in the physical ability of pygmy squid to escape a predator, but instead changes in the types of escape responses utilised. This change in the decision-making process could be attributed to altered function of neurotransmitter receptors, such as $GABA_A$ receptors, as suggested previously for molluscs (Watson *et al*., 2014; Moya *et al*., 2016) and fishes (Nilsson *et al*., 2012; Lai *et al*., 2015; Heuer and Grossell, 2014; Schunter *et al*., 2018). Changes to visual acuity (Chung *et al*., 2014), the function of the lateral line systems (see York and Bartol, 2014), or other alterations to sensory inputs, could also contribute to changes in predatory behaviour, either through impaired neurotransmitter function, or possibly other mechanisms (Briffa *et al*., 2012). The alterations to predatory behaviours and strategies that occurred at elevated CO_2 for both squid species would presumably provide prey with a higher chance of escape in the wild. Another reason that I did not observe differences in capture success could be because the experiment was carried out in a confined arena, where the prey did not have the full opportunity of escape that they may have in the wild. Therefore, it is possible that the results of first strike capture success of prey at elevated CO_2 could be different in the wild compared to those observed here, but field-based studies would be required to test this hypothesis.

As expected, there was a positive correlation between body size (mantle length) and activity levels in bigfin reef squid. There was also a significant effect of CO_2 treatment on activity, with increases in the total distance moved, average speed, and time spent active by bigfin reef squid under elevated CO_2. This increased activity closely resembles the effects of elevated CO_2 on pygmy squid activity levels (Spady *et al.*, 2014) but contrasts to the results demonstrated by Zakroff *et al.* (2017) for paralarvae of the longfin inshore squid. Decreased

activity reported by the latter study could potentially be associated with inhibited development of the aragonitic statolith in early life stages (Kaplan *et al.*, 2013), which controls for motion balance and orientation in cephalopods (Messenger, 1970; Arkhipkin and Bizikov, 2000). Both the current study, and Spady *et al.* (2014), exposed squid to elevated CO_2 after the statolith was fully developed. It would be valuable to test the activity levels of pygmy squid and bigfin reef squid after exposure to elevated CO_2 during early development to test this hypothesis. Another possible explanation for the differences in activity responses to elevated CO_2 between studies could be the different life stages used, as the dynamics of swimming can be very different when comparing paralarvae to juveniles or adults. Paralarvae usually have relatively smaller fins and larger funnel apertures, whereas juvenile and adult squid have relatively larger fins and smaller funnel apertures as well as more streamlined bodies (Bartol *et al*., 2008). Differences in physiology among species, latitudinal location, and types of environments they are exposed to could also play a role in the activity level response to elevated CO_2.

As squids rely heavily on cryptic behaviours to avoid detection by predators (Hanlon & Messenger, 2018), an increase in activity in the wild could be significant, as immobility is a major factor in effective crypsis, and most predators are sensitive to motion in their visual fields (Curio, 1976). It is possible, therefore, that higher activity resulting from elevated CO_2 could lead to higher levels of predator induced mortality in the wild as demonstrated in juvenile coral reef fish (Munday *et al.*, 2010). However, this would be difficult to investigate in the genus *Sepioteuthis* as their home range is 100s of kilometres (Pecl *et al.*, 2006), making field studies difficult. Furthermore, because these squid are less habitat associated and more pelagic than reef fish, the increase in activity seen here may not be as consequential on the

risk of predation. Regardless, higher levels of activity could still adversely affect the delicately balanced energy budgets of squids (Rodhouse, 1998).

This study demonstrates that there are similar behavioural effects of elevated CO_2 on two separate cephalopod orders that occupy largely distinct niches, and that these effects are consistent with those seen in a range of other taxa (Allan *et al.*, 2013; Pistevos *et al.*, 2015; Watson *et al.*, 2017). The similar effects of elevated CO_2 on both species seen here indicates potential for a variety of cephalopods to be adversely affected, with possible consequences for a diversity of marine habitats and ecosystems. One important consideration, however, is that squids have relatively short lifespans, rapid growth rates, large populations, and a high intrinsic rate of population increase which may also afford them a high potential to adapt to changes in the physical environment (Boyle & Rodhouse, 2005). Selection for genotypes more tolerant to elevated CO_2 conditions (Munday *et al.*, 2012) could potentially lead to squids being able to cope with future oceanic conditions. Therefore, future studies should investigate the potential for adaptation of cephalopod behaviour to elevated CO_2.

Chapter 5: Elevated carbon dioxide does not affect learning and conditional discrimination in the bigfin reef squid, *Sepioteuthis lessoniana*

Blake L. Spady, Philip L. Munday, Sue-Ann Watson

5.1 – Summary

Cephalopods have some of the most advanced cognitive abilities among invertebrates and are one of the few invertebrate taxa to demonstrate conditional discrimination. Any impacts to their learning ability could have far-reaching consequences for their populations and the communities they inhabit. Projected future carbon dioxide (CO_2) levels have adverse effects on learning in some fishes; however, the effects of elevated CO_2 on cephalopod learning has not been investigated. Here, I test the effects of elevated CO_2 (1,084 µatm) on learning and the capacity for conditional discrimination in the bigfin reef squid (*Sepioteuthis lessoniana*), a species which has previously demonstrated altered behaviour as a result of elevated CO_2. A three-task experiment within a two-choice arena was used to test learning and conditional discrimination. Learning was measured by improvements in task completion in repeated trials over time and the number of trials needed to pass each task. Squid exhibited significant learning ability, with a decrease in the number of trials needed to complete each successive task in repeated trials, but learning was not affected by elevated CO_2. There was no effect of CO_2 treatment on the number of trials needed to pass tasks, time to complete each trial, or in the percentage of correct choices within each task. Half of the squid in both CO_2 treatments demonstrated conditional discrimination by completing all three tasks. There was no evidence that elevated CO_2 affected conditional discrimination in *S. lessoniana*. Notably, this

is the first study to demonstrate conditional discrimination in any species of the order Teuthida. While it is not clear why some ecologically important behaviours are altered by elevated CO_2 whereas others are unaffected in the same species, these results provide no evidence that squid learning or conditional discrimination is affected by future projected CO_2 levels.

5.2 – Introduction

The coleoid cephalopods (squids, cuttlefishes, and octopuses), when compared to other invertebrates, show remarkably advanced learning and memory abilities (Dickel *et al.*, 1999). Their complex nervous systems and highly diverse behaviours have been compared to that of lower vertebrates, despite considerable evolutionary distance between these taxonomic groups (Boycott & Young, 1950; Boycott, 1961; Hanlon & Messenger, 2018). The morphology, physiology, ecology, and behaviours of coleoid cephalopods (henceforth referred to as cephalopods) were shaped by a coevolutionary arms race with modern teleost fishes (Mather & Kuba, 2013), resulting in many abilities and traits that both groups share (Packard, 1972; O'Dor & Webber, 1986). Among these are advanced eyes, large brains, and a capacity for visual conditional discrimination (Hvorecny *et al.*, 2007; Mather & Kuba, 2013).

Conditional discrimination is the ability to control discrimination through a sensitivity to context, which has significant benefits to individual performance (Mackay, 1991). For example, if reinforcement is delivered when an individual presses a square button and not a triangular button, there is discrimination. However, if reinforcement is contingent upon pushing the square button only after hearing the auditory stimulus "square", there is conditional discrimination. In this example, the auditory stimulus is the conditional stimulus,

and the square button is the discriminative stimulus. Aside from cephalopods, conditional discrimination has only been demonstrated in two other invertebrate species, the sea hare, *Aplysia californica* (Colwill *et al.*, 1988), and the honeybee, *Apis mellifera* (Couvillon & Bitterman, 1988; Brown *et al.*, 1998). High level learning in cephalopods undoubtedly contributes to their continued ecological success, benefiting their hunting, predator avoidance, social interactions, and navigation (Hanlon & Messenger, 2018).

Cephalopods, and squid in particular, occupy an important central role in marine food webs as they prey upon a wide range of marine species and are predated on by an even greater range of species (Hanlon & Messenger, 2018). The use of discrimination is important for both hunting and predator avoidance. Squids and cuttlefishes detect prey predominately by sight and hunt with a wide array of techniques that vary depending on prey type (Boycott, 1954; Ross, 1971; Curio, 1976; Ross & Von Boletzky, 1979). They also use subtle visual cues to discriminate weaker or slower individuals within schooling fishes, ensuring greater capture success (Neill & Cullen, 1974). Furthermore, learning to identify predators can influence survival as predator recognition is not always innate knowledge (Brodie Jr *et al.*, 1991; Mitchell *et al.*, 2011). Animals can also fine tune the efficiency of their anti-predator responses by learning which predators are a substantive threat and which are not (Ferrari & Chivers, 2011).

Discrimination also supports spatial orientation, especially in benthic associated species (Hvorecny *et al.*, 2007). Advanced conditional discrimination and long-term memory has been demonstrated in a number of cephalopod species (e.g., octopuses and cuttlefishes) using maze experiments (Schiller, 1949; Wells, 1964, 1967; Walker *et al.*, 1970; Boal *et al.*, 2000;

Karson *et al.*, 2003; Alves *et al.*, 2006, 2007; Hvorecny *et al.*, 2007). Compared to laboratory experiments, conditional discrimination supporting spatial orientation in the wild often differentiates between much more specific and subtle cues within an unstable environment (e.g., Forsythe & Hanlon, 1997). The importance and level of complexity of spatial learning among species depends on their lifestyle and habitat type, but is expected to be widespread among animals (Capaldi *et al.*, 1999).

Anthropogenic activity has resulted in an increase in atmospheric concentrations of CO_2 by more than 40% since the industrial revolution and the oceans absorb about a third of all atmospheric CO_2 (Dlugokencky & Tans, 2018). If CO_2 emissions continue to be released at the current rate (RCP8.5), atmospheric CO_2 could exceed 900 ppm by the end of this century (Collins *et al.*, 2013). Furthermore, seasonal fluctuations in the partial pressure of CO_2 (pCO_2) are projected to be amplified with increasing CO_2 concentrations due to the increased Revelle (buffer) factor of acidified seawater (McNeil & Sasse, 2016). CO_2 concentrations matching these worst case scenarios for the end of this century elicit a range of altered behavioural responses in a variety of marine taxa (Clements & Hunt, 2015; Nagelkerken & Munday, 2016), including inhibited learning (Ferrari *et al.*, 2012; Jutfelt *et al.*, 2013; Chivers *et al.*, 2014).

Recent studies show that elevated CO_2 affects predator avoidance and prey capture behaviours in two species of tropical squid. The two-toned pygmy squid, *Idiosepius pygmaeus*, responds to elevated CO_2 with altered escape responses (Spady *et al.*, 2014). Furthermore, *I. pygmaeus* and bigfin reef squid (*Sepioteuthis lessoniana*) respond to elevated CO_2 with altered predatory behaviours, such as increased latency to attack prey, and increased activity (Spady *et al.*, 2014, 2018, **Chapter 4**). Both in their predatory and anti-

predator responses, these species displayed altered body pattern choice at elevated CO_2 (Spady *et al.*, 2014, 2018, **Chapter 4**).

Although the effects of elevated CO_2 on learning have not been investigated in any invertebrate species, elevated CO_2 does inhibit learning in some fishes. The tropical damselfish, *Pomacentrus amboinensis*, failed to learn to appropriately respond to a common predator fish after four days exposure to elevated CO_2 (Ferrari *et al.*, 2012). In the temperate fish, *Gasterosteus aculeatus*, learning and memory is also affected by elevated CO_2 (~1,000 µatm), with reduced arena escape times over successive trials (Jutfelt *et al.*, 2013). The effects of elevated CO_2 on learning in reef fishes have been linked to impaired function of $GABA_A$ receptors, a major inhibitory neurotransmitter receptor (Chivers *et al.*, 2014). In squids, $GABA_A$-like receptors and other inhibitory mechanisms are also important for learning processes (Conti *et al.*, 2013). For example, $GABA_A$ is likely to be involved in the experience-dependent learning of prey capture and escape responses, which is dependent on the inhibitory control of the giant synapse output (Preuss & Gilly, 2000). While the relationship between CO_2 induced altered behaviour and $GABA_A$ has not been investigated in squids, the effects of elevated CO_2 on the predator escape responses in the gastropod mollusc, *Gibberulus gibberulus gibbosus*, has been linked to interference with $GABA_A$-like receptors (Watson *et al.*, 2014), which suggests that learning could be affected by elevated CO_2 in molluscs.

In this study, I investigate the effects of elevated CO_2 on the learning and capacity for conditional discrimination in the bigfin reef squid, *S. lessoniana*. Although conditional discrimination or associative learning has not been previously demonstrated in a species from

the order of *S. lessoniana* (Teuthida), a wide range of cephalopod species appear to share advanced learning capabilities (Boal, 1996; Karson *et al.*, 2003; Hvorecny *et al.*, 2007; Zepeda *et al.*, 2017). Furthermore, while conditional discrimination has not been demonstrated in many invertebrate species, it does occur in the relatively simple-brained sea slug, *Aplysia californica* (Colwill *et al.*, 1988). Therefore, conditional discrimination is likely to be more widespread throughout the animal kingdom than currently recognised.

The bigfin reef squid was chosen due to their close association with coral reef habitats (Norman, 2003), indicating a high prioritisation of spatial orientation when compared to more pelagic squid species. *S. lessoniana* is a very active species and has one of the largest global distributions of any inshore squid (Jereb & Roper, 2005). The genus *Sepioteuthis* has been noted for their complex learned social and mating behaviours (Moynihan & Rodaniche, 1982; Hanlon & Messenger, 2018; Sugimoto & Ikeda, 2012). Furthermore, the previously demonstrated effects of elevated CO_2 on predatory behaviours make *S. lessoniana* an ideal subject for this study.

I hypothesised that, given previously demonstrated altered behaviours at elevated CO_2, the learning and potential conditional discrimination abilities of *S. lessoniana* may be impaired by elevated CO_2 exposure. To test this, I subjected control and elevated CO_2 exposed squid to repeated trials in a two-choice arena experiment with three separate tasks, closely following the methods of Karson *et al*. (2003) and Hvorecny *et al*. (2007) used for demonstrating conditional discrimination in cuttlefishes. In the current experiment, squid were trained using visual cues in repeated motivation-based trial and error tasks with the goal of using the designated exit. Learning was measured as improvements in task completion over time and

the number of trials needed to pass each task. By completing all three tasks, squid will have demonstrated conditional discrimination. If learning or the conditional discrimination capabilities of *S. lessoniana* are altered by elevated CO_2, this could adversely affect their prey capture, predator avoidance, navigation, migration, and social interactions.

5.3 – Methods

CO_2 treatment systems

Experiments were conducted using 8,000 L recirculating seawater systems at James Cook University's research aquarium in Townsville, Australia. Experimental treatments were a current-day ambient control (502 µatm CO_2) and an elevated CO_2 treatment (1,084 µatm CO_2) consistent with upper end-of-century projections for RCP8.5 (Collins *et al.*, 2013). A custom-built pH control system dosed CO_2 into 3,000 L sumps to achieve the desired pH level in the elevated CO_2 treatment. Briefly, pH was measured continuously by an inline ISFET pH sensor (Tophit CPS471D, Endress+Hauser, Reinach, Switzerland) that communicated with a computerised controller (OMNI C40 BEMS, Innotech, Brisbane, Australia) and regulated the CO_2 dosing. pH on the NBS scale (pH_{NBS}) was measured daily (Seven2Go Pro, Mettler Toledo, Switzerland) and dosing set points were adjusted as necessary to maintain the target pCO_2 in each treatment. Equilibrated seawater from each system was delivered at a rate of 1.5 L min^{-1} to tanks containing squid. Temperature was measured daily in each tank (Comark C26, Norfolk, UK).

Water samples were taken weekly to determine pH on the total scale (pH_T) by spectrophotometry (UVmini-1240, Shimadzu, Suzhou Instruments Co. Ltd., Kyoto, Japan) using m-cresol purple as an indicator dye (Dickson & Millero, 1987; Dickson *et al.*, 2007). Total

alkalinity was estimated weekly by Gran Titration (888 Titrando Metrohm AG, Switzerland) ensuring titration calibrations remained within one percent of certified reference material from Dr. A.G. Dickson (Scripps Institution of Oceanography, batch #135). Salinity was measured weekly using a conductivity sensor (HQ15d, Hach, Loveland, CO, USA). Carbonate chemistry parameters (**Table 5.1**) were calculated in CO2SYS (Pierrot *et al.*, 2006) using the constants K1, K2 from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and Dickson *et al.* (2007) for $KHSO_4$.

Table 5.1 – Seawater carbonate chemistry – The temperature, salinity, $pH_{(T)}$, total alkalinity, and pCO_2 for each treatment. Values are means ± SD.

Treatment	Temperature (C°)	Salinity	$pH_{(T)}$	Total alkalinity (µmol kg^{-1} SW)	pCO_2 (µatm)
Control	26.8 (±0.8)	36.1 (±0.7)	7.98 (±0.03)	2464 (±65)	502 (±26)
Elevated-CO_2	26.5 (±0.5)	35.8 (±0.5)	7.68 (±0.06)	2345 (±123)	1084 (±155)

Animal collection and care

Bigfin reef squid (85-192 mm mantle length) were collected by dipnet in May and June 2017 from the Townsville breakwater, Queensland, Australia. Animals were captured at night with a 2.5 cm mesh, round frame dip-net and transported immediately to James Cook University, Townsville. Squid were kept individually in round tanks (47Ø x 51H cm) filled to 67-litres with a continuous flow of fresh seawater. Individuals were kept at control conditions for 14 days before being transferred to separate tanks of the same size that received a continuous flow of either control or elevated CO_2 treatment water. Squid were fed a variety of live fishes and prawns three times daily. Individuals were maintained at either control (n=6) or elevated CO_2 (n=6) for 40 days before learning trials were initiated. This treatment duration represents

approximately 20% of the species' average 208-day lifespan in the wild (Walsh *et al.*, 2002). Mantle length (± 1 mm) of individuals were measured at the conclusion of the experiment.

Testing arena

The testing arena and procedures for this experiment closely resemble that of Karson *et al.* (2003) and Hvorecny *et al*. (2007). A circular testing arena (56Ø x 40H cm) was constructed from dark grey PVC (**Fig. 5.1**). As with the experiments of Karson *et al.* (2003) and Hvorecny *et al*. (2007), this is a two-choice exit arena, providing visual cues (object cues and exit door frame) to teach individuals which exit is open and which is closed. At one end of the arena was a door blocking off the main arena from a rectangular habituation chamber (27 x 15 cm) in which the animal began the trial. Upon entering the centre of the arena from the habituation chamber, the animal could view two exits (20 cm diameter and raised 10 cm from the base of the arena) located directly to the left and to the right. When utilised, visual object cues were placed directly opposite the habituation chamber door. The arena and surrounding tank were filled with water to 29 cm deep.

Attached by Velcro to each exit was a laminated frame. One frame was solid yellow in colour while the other was yellow with black stripes. These two frames were alternated between the left and right exit for different individuals. A piece of clear Plexiglass™ that did not distort polarization (Shashar *et al.*, 1996) was used to block the desired exit from the outside of the arena. Blocked exits were used to train squid to use a particular exit given a specific visual cue. In the experiments of Karson *et al*. (2003) and Hvorecny *et al*. (2007), an arena with a transparent bottom was lifted above the tank floor in an attempt to encourage cuttlefish, which preferred staying on the substrate, to exit the arena to reach the floor. In this

experiment however, preliminary tests of squid revealed an adverse reaction to the dark enclosure of the arena and a preference for areas of the tank with no obstructions above them. I instead used this as motivation for squid to escape the enclosed arena by navigating out one of the two exits. Therefore, a plywood board covered the top of the arena, while areas surrounding the arena were open and not covered. Trials were viewed via the display of a video camera (Panasonic HC-V160) which filmed downwards through a hole in the plywood board.

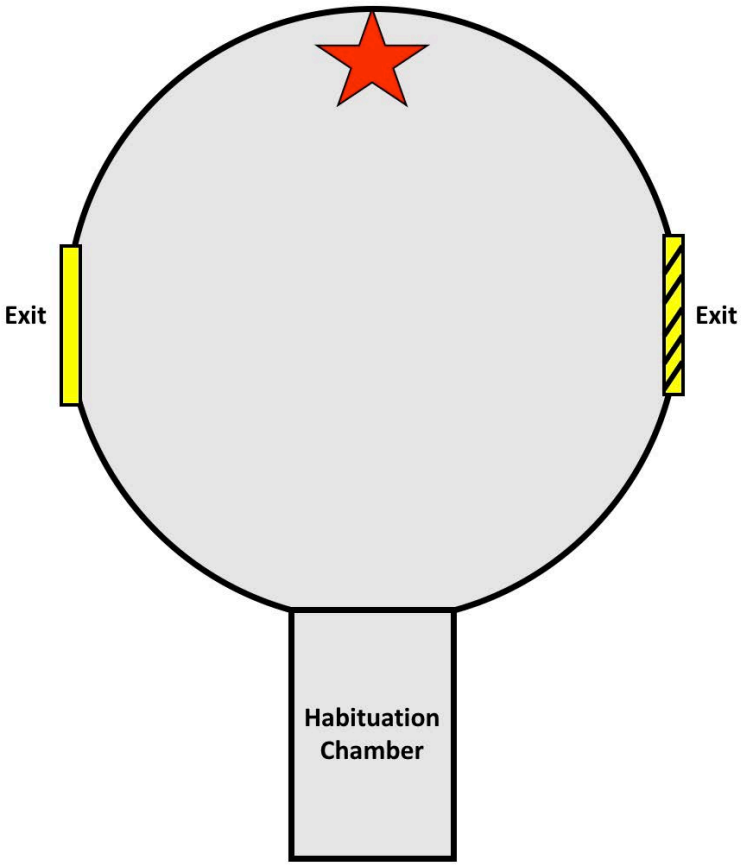

Figure 5.1 – Testing arena as viewed from above. Red star indicates location of visual object cue (either brick or plastic algae). Exits were placed on the left and right side of the arena and were fitted with a visual frame for reference (either solid yellow or yellow with black stripes). The habituation chamber included a rising gate that allowed squid to enter the arena after the habituation period.

Learning trails

-General procedures

At the beginning of each trial, squid were placed in the habituation chamber for one minute, after which the door to the arena was opened. Preliminary trials determined that a one-minute habituation time was enough for squid to resume normal behaviour and swimming postures when entering the arena. Squid were given 30 seconds to leave the habituation chamber and enter the arena. At the end of this period of time, if they did not enter the arena, they would be gently guided out of the habituation chamber with a dip net. All squid had to be guided out of the habituation chamber at least once during preliminary trials. After exiting the habituation chamber, the door was closed behind them.

Upon entering the arena, each animal was given five minutes to exit; if they did not exit the arena within this time, they were guided out of the correct exit with a dip net. To encourage squid to exit the arena, a dip net was placed in the arena directly in front of the habituation chamber door 20 seconds after the animal entered the arena. After an additional 10 seconds, the net was lifted up and down at a set rate (full up/down cycle every two seconds), using a stop watch, until the squid had exited the arena. For each of the three tasks, individuals were given a maximum of 60 trials per task to successfully pass and proceed to the next task. If a squid could not pass a task within the 60 trials, they would be marked as “failed” and would not continue on to the subsequent task. Criteria for passing tasks are described in the following sections. Individuals performed up to seven trials per day with an inter-trial interval of 45 minutes. Animals were tested in their treatment water.

-Preference testing

During preference testing, both exits were unobstructed and there was no visual object cue. Preference testing was complete when an individual had: (a) exited the maze in under three minutes in six out of seven consecutive trials, and (b) used both the left and right exit at least once. Preference was defined as the side (left or right) through which the squid passed through most frequently in these six trials (Karson *et al.*, 2003; Hvorecny *et al.*, 2007). If both exits were used equally, another trial would be performed to determine preference. A preference was considered strong if individuals chose the same exit in five or more of the trials.

-Tasks 1 & 2: Learning

In Task 1 (testing against preference), individuals were tested against their preferred exit, meaning the exit they preferred in preference testing was blocked off with the Plexiglass barrier. During this task, an object cue (either a red brick or a green plastic algae) was placed directly opposite the entrance from the habituation chamber. For Task 1, half of the animals from each treatment were presented the red brick as a visual cue, while the other half were presented the green plastic algae. This arrangement provided squid with two visual indications to learn which doorway was open: the object cue and the coloured frames around the exits.

To successfully pass Task 1, as well as subsequent tasks, individuals were required to exit the arena in less than three minutes in six out of seven consecutive trials without first attempting to pass through the blocked exit by touching the Plexiglass barrier with their mantle. Once Task 1 was completed, the opposite object cue replaced the initial one within the arena, and

the opposite exit was blocked (Task 2 - testing with preference). Aside from the difference in visual object cue and which exit was obstructed, Task 2 followed the same procedures as in Task 1. By analysing improvements in correct exit choice and time to exit the arena over successive trials within tasks, as well as number of trials performed before passing each task, I was able to compare learning performances between individuals and CO_2 treatments.

-Tasks 3: Conditional discrimination

Finally, after Task 2 was completed, squid were tested in trials in which the presented object cue (and the corresponding blocked door) was semi-randomised (Task 3) by flipping a coin, but ensuring that the same object cue was not presented more than four times consecutively. In Task 3, the squid had to determine which exit to use based on learned knowledge from the previous tasks and distinguish which exit and frame would not be blocked by taking note of the object cue presented. By successfully choosing the correct exit in six out of seven consecutive trials, before the failure cut-off of 60 total trials, individuals will have demonstrated conditional discrimination by controlling their exit choice by a sensitivity to the context of presented visual cues.

Statistical analyses

Statistical analyses were performed using R statistical software (R Development Core Team, 2018). To analyse the effect of CO_2 treatment on individual ability to pass each task, a generalised linear model (GLM) with a binomial distribution was used. Here, preference strength (number of exits through preferred side during preference testing) and visual cue were used as explanatory fixed factors. To determine the effect of elevated CO_2 on number of trials needed to pass each task, a GLM with a Poisson distribution was used. The difference

in average time to exit the arena in each task was compared between CO_2 treatments with a GLM with a Gaussian distribution and a log link.

Repeated measures generalised linear mixed-effects models (GLMM) with negative binomial distributions were used to analyse trends in time to exit the arena over successive trials within each task. Repeated measures GLMMs with binomial distributions were used for trends in the successful exit choice over successive trials. These repeated measures analyses included mantle length and preference strength as explanatory variables and squid ID as a random effect. Repeated measures GLMMs were also used to compare the differences in mean time to exit the arena (Gaussian with log link), number of trials needed to meet passing criteria (Poisson distribution), and percent of correct exit choices (Gaussian with logit transformation) among tasks, as well as to examine effects of CO_2 treatment on these relationships. These repeated measures analyses included preference strength as an explanatory variable and squid ID as the random effect.

Differences in preference strength between CO_2 treatments was analysed with a linear regression model. The effect of average time taken to exit the arena in Task 1 and the ability to successfully reach Task 3, as well as the effect of number of trials needed to pass Task 1 on the ability to reach and complete Task 3, were analysed with a GLM with a binomial distribution. Residual analysis indicated that data met the assumptions of normality and homogeneity of variance given the specified distributions chosen. A Cohen's d power analysis was performed on the probability of finding an effect of treatment on the ability for individuals to successfully demonstrate conditional discrimination.

5.4 – Results

Six of the twelve squid tested successfully completed all three tasks, indicating that *S. lessoniana* has a capacity for conditional discrimination. Three of these squid were from the control treatment and three were from the elevated CO_2 treatment (GLM, χ^2=0.024, p=0.876). All squid completed preference testing, taking between 9-30 trials. Four squid from control exhibited a strong preference towards one exit (5-6 uses of the same exit) and two squid from the elevated CO_2 treatment exhibited a strong preference towards one exit (GLM, χ^2=1.000 p=0.317). All twelve animals met the passing criteria for Task 1, taking between 7-38 trials. Seven individuals met the passing criteria for Task 2; four of these individuals were from the control treatment and three were from the elevated CO_2 treatment (GLM, χ^2=0.707, p=0.401). Of the seven squid to reach Task 3, one individual from the control treatment failed to meet the passing criteria for Task 3 and no squid from the elevated CO_2 treatment failed to meet the passing criteria for this task (GLM, χ^2=1.243, p=0.265).

The mean time for individuals to exit the arena in Task 1 (testing against preference) had a clear positive relationship with their ability to reach Task 3 (testing for conditional discrimination) (GLM, χ^2=6.705, p=0.010). Individuals that successfully reached Task 3 had an average exit time in Task 1 trials of 32.1 secs ($\pm$ 5.2 SE), whereas individuals that did not reach Task 3 spent only 10.1 secs ($\pm$ 4.9 SE) in the arena during Task 1 trials. There was also a positive relationship between the number of trials performed to pass Task 1 and the ability to reach and pass Task 3 (GLM, χ^2=4.053, p=0.044). The mean number of trials needed to pass Task 1 in individuals that reached and passed Task 3 (20.8 $\pm$ 4.2 SE) was nearly double that of

individuals that failed to reach and pass Task 3 (11.3 ± 2.5 SE). All subsequent results focus only on individuals that successfully reached and passed Task 3.

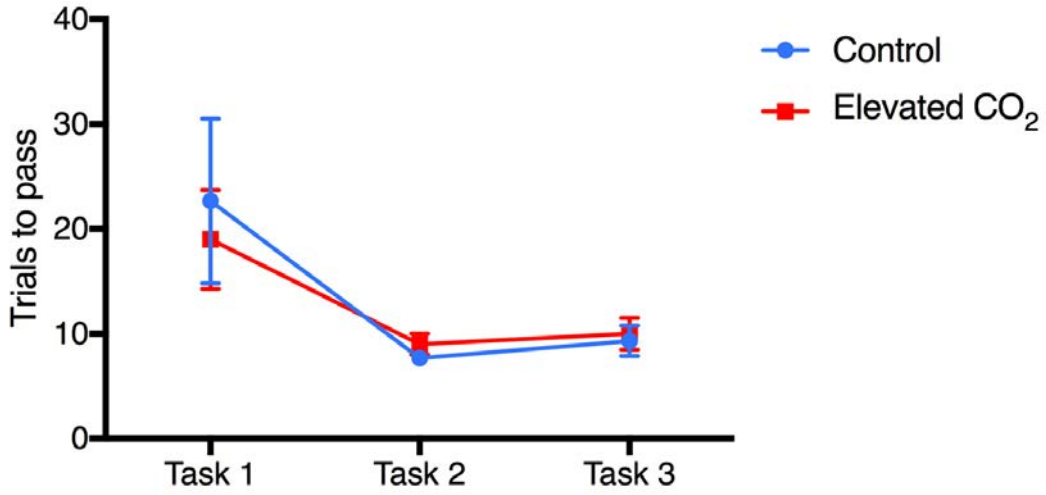

Figure 5.2 – The mean number of trials (± SE) performed before successfully meeting the passing criteria for each task in control and elevated CO_2 squid.

The number of trials needed to successfully complete each task did not differ between CO_2 treatments (**Fig. 5.2**): Task 1 (GLM, χ^2=0.969, *p*=0.325), Task 2 (GLM, χ^2=0.320, *p*=0.571), Task 3 (GLM, χ^2=0.069, *p*=0.793). There was also no difference between CO_2 treatments in the mean time to exit the arena within each task (**Fig. 5.3**): Task 1 (GLM, χ^2=0.011, *p*=0.916), Task 2 (GLM, χ^2=0.497, *p*=0.481), Task 3 (GLM, χ^2=0.382, *p*=0.537). Improvements in exit times and correct exit choices of squid over successive trials within each task did not significantly differ between CO_2 treatments. In Task 1, the time for squid to exit the arena did not significantly decrease over successive trials (LME, χ^2=0.426, *p*=0.514), and there was no effect of CO_2 treatment on this relationship (LME, χ^2=0.909, *p*=0.340). There was an improvement in correct exit choice for squid over successive trials in Task 1 (GLMM, χ^2=5.806, *p*=0.016), but

this relationship was not influenced by CO_2 treatment (GLMM, χ^2=0.073, p=0.787). There was no significant improvement in exit time in Task 2 over successive trials for squid (LME, χ^2=0.431, p=0.511) and no effect of CO_2 treatment on this relationship (LME, χ^2=2.499, p=0.114). Similar to Task 1, there was also a significant improvement in Task 2 in the correct exit chosen over successive trials (GLMM, χ^2=6.617, p=0.010), but CO_2 treatment had no effect on this relationship (GLMM, χ^2=1.960, p=0.162). In Task 3, the time to exit the arena over successive trials for all squid did not improve (LME, χ^2=1.499, p=0.221) and there was no effect of CO_2 treatment (LME, χ^2=2.005, p=0.157). While there was a trend in Task 3 of increasing correct choices over successive trials, this was not significant (GLMM, χ^2=3.117, p=0.078) and there was again no effect of elevated CO_2 (GLMM, χ^2=0.018, p=0.892).

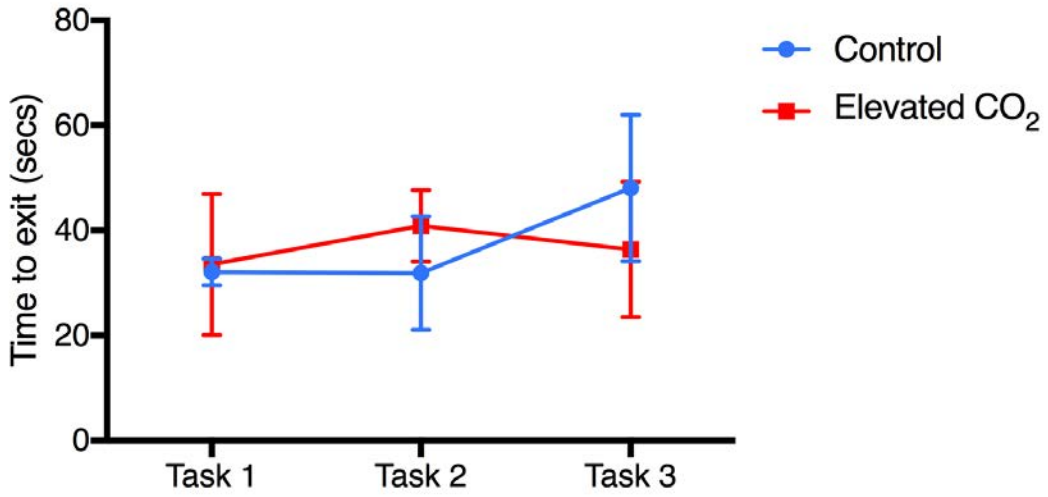

Figure 5.3 – The mean exit time (± SE) in successful trials for each task in control and elevated CO_2 squid.

There was a significant decrease in the number of trials needed to complete each task from Task 1 ($\bar{X}$ = 21 trials) to Tasks 2 and 3 ($\bar{X}$ = 8 and 10 trials, respectively) (GLMM, χ^2=40.842, p<0.001) and elevated CO_2 had no effect on this relationship (GLMM, χ^2=1.249, p=0.535). The mean time to exit the arena did not change significantly among Tasks 1, 2, and 3 (LME,

χ^2=0.165, p=0.315), and there was also no effect of elevated CO_2 (LME, χ^2=2.594, p=0.273). The percentage of trials within each task in which individuals chose the correct exit increased from Task 1 (71% correct) to Tasks 2 and 3 (84% and 80% correct, respectively) but this was not significant (GLMM, χ^2=2.919, p=0.232) and did not differ between CO_2 treatments (GLMM, χ^2=2.103, p=0.350) (**Fig. 5.4**). A power analysis revealed a power of 0.221 on the effect of treatment on the ability to demonstrate conditional discrimination.

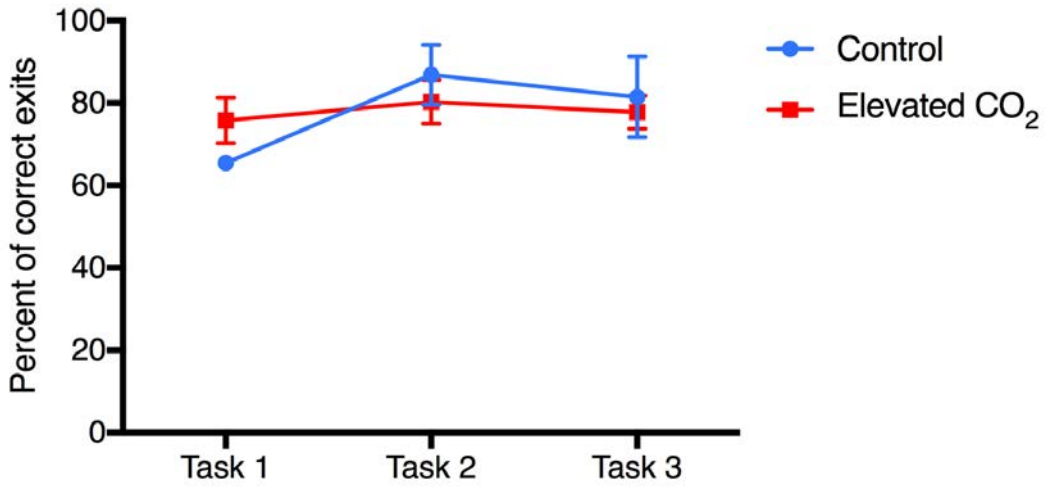

Figure 5.4 – The mean percentage of trials (± SE) in which squid chose the correct exit within each task for control and elevated CO_2 squid.

5.5 – Discussion

Of the twelve bigfin reef squid (*S. lessoniana*), that participated in the experiment, six of them completed Task 3. This, to my knowledge, is the first demonstration of conditional discrimination in a teuthid cephalopod. The six squid that displayed conditional discrimination were represented by three individuals from control and three individuals from elevated CO_2, indicating that elevated CO_2 had no effect on the capacity of this species to conditionally discriminate. Furthermore, elevated CO_2 did not have an effect on any of the traits measured

within this experiment, such as the number of trials to complete each task, time to exit the arena, and percentage of correct exit choices within each task. This suggests that elevated CO_2 also has no effect on the learning or long-term memory of *S. lessoniana*. This experiment has brought to light some of the remarkable cognitive abilities of *S. lessoniana*, and shown that seawater pCO_2 projected for the end of the century under a worst case scenario does not inhibit these abilities.

Squid in this experiment demonstrated individual variation in their learning and conditional discrimination abilities, and this variation was greater than any effect of CO_2 treatment. Importantly, individuals that passed all three tasks spent more time in the arena during each trial in Task 1 compared to individuals that failed to reach and pass Task 3. It is possible that spending more time within the arena gave the squid time to better assess their surroundings and commit visual cues to memory. Animals that failed to reach Task 3 were also more erratic in their Task 1 escapes, and appeared stressed in comparison to individuals that demonstrated conditional discrimination. Throughout the experiment, squid that demonstrated conditional discrimination would usually slowly enter the arena and then make a choice after a brief pause somewhere between the two exits. In contrast, individuals that did not reach or pass Task 3 would often rapidly jet out of the habituation chamber and then repeatedly jet around the perimeter of the arena until reaching an exit. Due to this, during Task 2, these individuals had a mean percentage of correct exit choices of 51%, indicating complete randomness in their exit choice. Individuals that demonstrated conditional discrimination chose the correct exit in Task 2 in a mean of 84% of their trials. This high percentage of success, along with the significant increase in correct exit choice over

successive trials, suggests that these individuals learned from their incorrect choices during the initial trials within the task and remembered the correct exit.

Of the seven squid to reach Task 3, four were from the control treatment. However, one squid from control did not demonstrate conditional discrimination despite having reached Task 3. This squid, like individuals that failed Task 2, successfully chose the correct exit in only 50% of 26 total trials during Task 2. It seems likely that this individual was choosing the exit at random in Task 2 and met the passing criteria by chance. The lack of conditional discrimination among visual cues in this individual becomes apparent in the randomised trials of Task 3, in which only 35% of 60 trials did it choose the correct exit. Among animals that successfully demonstrated conditional discrimination, the mean percentage of correct exit choices was only slightly lower in Task 3 (80%) compared to in Task 2 (84%). A decrease in percentage of correct choices here was expected as the random aspect of Task 3 was a new concept for the squid. However, this difference was not as large as expected and shows advanced long-term memory and context dependent discriminatory abilities.

Individuals that did not demonstrate conditional discrimination took half as many trials to complete Task 1 as those that did demonstrate conditional discrimination by passing Task 3. It appears that performance during Task 1 was crucial for the squid to successfully reach and pass Task 3. A faster exit time and fewer number of trials for each task would seemingly indicate better performance. However, in this experiment it seemed important for individuals to spend time within the arena as well as to make a fair number of incorrect choices during Task 1 in order to understand the conditions of the arena. A drawback of this experiment is that once an individual passed Task 1, it was not presented that combination of visual cue and

corresponding exit again until after it successfully passed Task 2 and reached the random trials. Animals that passed Task 1 in a very short number of trials (some in the minimum 7 trials) may not have had sufficient trial and error experience in their learning process. Trial and error is an integral part of many learning processes, leading to sustained modifications of behaviour and improvements in complex tasks (Dayan & Balleine, 2002; Ruediger *et al.*, 2012). Squid that completed Task 1 in a minimal number of trials may have benefited from a reintroduction to Task 1 if they were unable to complete Task 2 after a set number of trials.

The ability for cephalopods to conditionally discriminate and rapidly learn, especially during early life, is important in order to keep up with their fast-paced lifestyle (Dickel *et al.*, 1999). Changes to these abilities could have adverse effects on squid predation, predator-avoidance, navigation/migration, and many other behaviours. This experiment provides no evidence that elevated CO_2 has any effect on the learning abilities of *S. lessoniana*. However, the small sample size, large variation in individual performance, and high difficulty of the passing criteria (50% pass rate) in this study may have masked any subtle effects of elevated CO_2 on learning. A power analysis revealed a probability of 22.1% of finding an effect if one were present. Perhaps with a larger sample size, a more easily achievable associative learning test, and memory reinforcement in the first tasks, potential differences in performance between CO_2 treatments would be more detectible. Nevertheless, this experiment demonstrates advanced learning capabilities in squid, and no detectible effect of adverse effects from elevated CO_2 exposure.

Adverse effects on learning arising from elevated CO_2 exposure in damselfish has been linked to inhibited $GABA_A$ neurotransmitter function (Chivers *et al.*, 2014). The effects of elevated

CO_2 on $GABA_A$ also appears to be responsible for a wide range of altered behaviours, such as in anti-predator responses in molluscs (Watson *et al.*, 2014) and anxiety, lateralisation, and olfactory preference in fishes (Nilsson *et al.*, 2012; Hamilton *et al.*, 2014; Heuer & Grosell, 2014; Lai *et al.*, 2015; Schunter *et al.*, 2018). Previously demonstrated effects of elevated CO_2 on behaviour in *S. lessoniana* include increased activity and altered predatory behaviours (Spady *et al.*, 2018, **Chapter 4**), but the mechanism responsible for these changes has not been investigated. The importance of $GABA_A$ receptors for learning in squids (Conti *et al.*, 2013), along with the lack of inhibited learning in the current experiment, suggests that previously demonstrated altered behaviours in *S. lessoniana* at elevated CO_2 (Spady *et al*., 2018) may be due to a different mechanism. It is important to investigate this mechanism in order to understand why some important behaviours are altered, whereas other equally important behaviours are not affected by elevated CO_2.

In addition to learning, the large relative size of cephalopod brains are needed for processing large amounts of sensory information (Young, 1988). Cephalopods can have 20 million receptor cells in the eye, requiring significant processing power from the brain and giving individuals a great deal of visual information to manage (Hanlon & Messenger, 2018). The ability to process complex visual cues, such as during male to male agonistic behaviour, is of great importance to their health and survival, as well as to the rewards of reproductive opportunities. As previous studies on fish have shown elevated CO_2 to inhibit learning via visual predator recognition (Ferrari *et al.*, 2012), perhaps testing predator recognition learning at elevated CO_2 would be a valuable approach for *S. lessoniana*. Also, as Ferrari *et al*. (2012) used pre-settlement juveniles to test learning, it could be worthwhile to investigate effects of elevated CO_2 on learning in *S. lessoniana* as juveniles rather than adults. In this way,

experiments could focus on naturally acquired learning during a life period in which learning is more rapid and ecologically beneficial.

While the current study investigates learning in support of spatial orientation rather than in support of predator recognition, Jutfelt *et al*. (2013) reported inhibited learning in fish in arena escape as a result of elevated CO_2 exposure. While both studies measured arena escape behaviours related to spatial orientation, there are a number of differences in the design of the studies and what they measured. Jutfelt *et al*. (2013) found reduced arena exit times in control fish from the initial trial to the second trial 20 days later, whereas fish held at elevated CO_2 showed no difference in exit times between the two trials. While this could be a measurement of learning, it could equally be a result of elevated CO_2 effects on exploratory behaviours (as the experiment was intended to measure), boldness, or simply activity levels. The present study is strictly an investigation into the learning of *S. lessoniana* with repeated motivation-based trial and error training, and provides no evidence of inhibited learning from elevated CO_2.

The importance of context learning in animals is of considerable ecological significance (Balsam and Tomie, 1985), and in squids, leads to the development of many important behaviours such as escape response, prey capture, body patterning, spatial orientation, social behaviours, and many more (Hanlon & Messenger, 2018). The results from this study indicate for the first time that squid of the order Teuthida are able to conditionally discriminate, further expanding the known range of taxa with this ability. Furthermore, I found no evidence for elevated CO_2 levels projected for the end of this century to have any effects on crucial learning behaviours of *S. lessoniana*. Future studies should investigate the effects of elevated

CO_2 on a wider range of learning behaviours and in a variety of cephalopod species to ensure that these cognitive abilities will remain unaffected under future ocean conditions.

Chapter 6: General Discussion

Climate change is occurring at a rate that may be too fast for some marine species to acclimate or adapt (Sunday *et al*., 2014). For populations to persist in rapidly changing conditions, individuals must be able to maintain their physiological performance, successfully reproduce, and compete with other species for resources. However, CO_2 levels projected for the end of this century have been shown to alter oxygen uptake, reproduction and development, behaviour, and learning in a variety of marine organisms (Nagelkerken & Munday, 2016; Cattano *et al*., 2018). These stressors could have serious effects on the ability of squids to maintain populations if they are similarly affected by elevated CO_2. The data chapters within this thesis provide novel investigations into the effects of elevated CO_2 on cephalopod aerobic performance, reproduction, early life history development, predatory behaviours, and learning. Together, these chapters demonstrate the degree of sensitivity of two cephalopod species from separate taxonomic orders to elevated CO_2 in a range of traits. The taxonomic separation between the species is important to note, as these results indicate that a wide range of cephalopods may respond to elevated CO_2 similarly.

6.1 – Effects of elevated CO_2 on squid

Physiological responses

The effects of elevated CO_2 on the aerobic performance of *Sepioteuthis lessoniana* and *Idiosepius pygmaeus* was investigated in **Chapter 2**, revealing tolerance in both species under elevated CO_2 conditions. Squid have been hypothesised to have blood with naturally low oxygen carrying capacity (O'Dor & Webber, 1986; Shadwick *et al*., 1990) and oxygen transport proteins (hemocyanins) that are extremely sensitive to changes in pH (Brix *et al*., 1989;

Bridges, 1995; Rosa & Seibel, 2008; Seibel, 2016). Therefore, small changes in blood pH could cause severe impairment of blood-oxygen binding. The pH sensitivity of blood-oxygen binding in squids suggests that exposure to elevated environmental CO_2 may have adverse effects on their oxygen uptake capabilities. Contrary to expectation, I found no effect of elevated CO_2 on the routine oxygen uptake rates, maximal oxygen uptake rates, aerobic scope, or recovery time in either species of squid. Therefore, both species appear to be tolerant to the direct effects of elevated CO_2 on oxygen uptake ability. Importantly, maximal oxygen uptake rates and aerobic scope were not affected, therefore the energetically expensive swimming mode will likely not be affected. Squids are strong acid-base regulators (Hu *et al*., 2011a, 2011b, 2013, 2014), which could explain why their aerobic performance is maintained under elevated CO_2. Furthermore, it was recently estimated that, in the absence of blood acid-base regulation, an increase in seawater pCO_2 to ~1,000 µatm would cause a drop in squid hemocyanin-O_2 saturation by no more than 1.6% (Birk *et al*., 2018). This small effect on blood-oxygen binding at climate change relevant CO_2 levels could explain why I observed no effects of ~1,000 µatm CO_2 on the two species of squid studied here.

My results contrast with some earlier studies that have found a significant effect of elevated CO_2 on oxygen uptake in large squids (e.g., Rosa & Seibel, 2008; Rosa *et al*., 2014). It is unclear why the oxygen uptake rates of some cephalopod species are affected by elevated CO_2 while others are not. Although it appears that some species are more susceptible to consequences regarding oxygen uptake from elevated CO_2 during early life-history stages (e.g., Gutowska *et al*., 2008; Sigwart *et al*., 2016), differences in exposure time to elevated CO_2 and measurement techniques may contribute to the variations in effects. Studies demonstrating negative effects of elevated CO_2 on oxygen uptake in adult cephalopods have ranged in

exposure time from just a few hours to a few days. In this study, *I. pygmaeus* and *S. lessoniana* were exposed to elevated CO_2 conditions for substantially longer, accounting for ≥20% of their natural lifespan. It is possible that this prolonged and more ecologically relevant exposure period allowed enough time to overcome any shock effects of elevated CO_2 exposure that could potentially indicate differences in oxygen uptake with shorter exposure durations.

Changes to the aerobic performance of squids could be especially consequential due to their highly active lifestyles and significant energy demands (O'Dor & Webber, 1986; Shadwick *et al*., 1990). The unaltered maximal oxygen uptake rates observed here suggest that elevated CO_2 has no adverse effects on the blood-oxygen binding capabilities of these species. Furthermore, the unaltered routine oxygen uptake rates indicate that their acid-base regulation is effective without compromising their energy budget to a degree in which it has an effect on aerobic performance. The increased costs of acid-base regulation from elevated CO_2 has been suggested to have the potential to reduce the amount of energy available to activities such as reproduction and growth (Dupont & Thorndyke, 2009). As optimal aerobic scope was maintained at elevated CO_2, there was no clear indication of increased costs from acid-base regulation observed in this experiment. It was therefore expected that energy allocated to reproduction and development would remain available under elevated CO_2 conditions.

In **Chapter 3**, the effects of parental exposure to elevated CO_2 on reproduction and early life-history development of *I. pygmaeus* were investigated. Elevated CO_2 exposure resulted in a decrease in the number of eggs per clutch, reduced embryo size, decreased distance between

eggs at spawning, and an increase in the swelling of eggs during late stage development. Despite the lack of effects of elevated CO_2 on the aerobic scope of *I. pygmaeus* adults in **Chapter 2**, there were direct effects of elevated CO_2 on their reproductive output. This indicates that the aerobic scope of an animal may not be an appropriate predictor of other aspects of their performance. Elevated CO_2 appears to be altering energy allocation in female *I. pygmaeus*, resulting in reduced reproductive output, but these changes to the energy budget may not be well captured by measurements of aerobic scope.

The negative effects of elevated CO_2 observed on embryos are seemingly due to parental exposure rather than from exposure of the eggs themselves. It is likely that *I. pygmaeus* is reducing the energy allocated towards reproduction in these unfavourable conditions in order to save energy for future reproductive events when preferred conditions return. However, if these conditions remain unfavourable, as would be the case with projected end-of-century CO_2 conditions, the large populations and high rate of population increase characteristic of the species could suffer. While the general capacity for any cephalopod to adapt to elevated CO_2 conditions is unknown, it remains hopeful that despite these changes to reproduction, *I. pygmaeus* will be able to adapt to higher CO_2 levels before the end of the century. The short lifespan of *I. pygmaeus* allows for the completion of >300 generations by the year 2100. This gives the species a large advantage in adaptive capacity and hopefully ample opportunity to cope with changing ocean conditions over the coming decades.

Behavioural responses

The behavioural responses of squid in this thesis were equally varied among traits as those observed in physiological responses to elevated CO_2. In **Chapter 4**, the effects of elevated CO_2

on the predatory behaviours of both *I. pygmaeus* and *S. lessoniana* were measured. A number of predatory strategies and behaviours in one or both species were altered at elevated CO_2 including latency to attack, striking distance, attack direction, and body pattern display. However, there was no effect of elevated CO_2 on learning in *S. lessoniana* (**Chapter 5**). While the changes to predatory strategies observed in **Chapter 4** had no influence on capture success in the confined arena that squid and prey were tested in, if these altered behaviours result in reduced prey capture in the wild, it could conflict with the high energy demands of squids. Furthermore, *I. pygmaeus* at elevated CO_2 were 20% less likely to attack their prey compared to control. As many squids have limited lipid storage capabilities (Moltschaniwskyj & Semmens, 2000), reductions in energy intake could have adverse effects on reproductive output as has been demonstrated in *I. pygmaeus* (Lewis & Choat, 1993). If reduced predation at elevated CO_2 leads to indirect adverse effects on reproductive output, this combined with the direct effects of elevated CO_2 on reproductive output observed in **Chapter 3** could be especially consequential for *I. pygmaeus*. This raises concerns about other potential indirect effects of elevated CO_2 that may alter squid behaviour and the ecosystems in which they inhabit.

Due to the key position of squid in marine food webs, changes to their predatory behaviours and strategies could have far-reaching effects. As the diets of squids consist of a wide range of taxa (Hanlon & Messenger, 2018), effects of elevated CO_2 on the predatory behaviours of squid could affect the populations of species across a wide range of ecosystems. The effects of elevated CO_2 on predator-prey interactions is an important area of research, but future studies need to place greater emphasis on these interactions when both predator and prey are exposed to elevated CO_2. A study with this focus on a tropical damselfish and a common

predator fish found reduced prey capture when the predator was exposed to elevated CO_2, but only when the prey was not (Allan *et al*., 2013). In other words, the performance of prey fish after exposure to elevated CO_2 was far more consequential when their predator was from the control CO_2 treatment. While this demonstrates that predator-prey interactions can be vastly different when both species are subjected to elevated CO_2 conditions, this is not to say that the effects seen in **Chapter 4** are likely to "balance out" once the prey species are also exposed to elevated CO_2. There are differences in tolerance to elevated CO_2 among species and as some species are severely impaired, other species are seemingly unaffected (e.g., Ferrari *et al*., 2011b). This differential tolerance is likely to lead to shifts in ecosystem structure through changes in competition with the more tolerant species becoming more dominant (McCormick *et al*., 2013).

The physiological mechanism responsible for these changes in squid behaviour are unknown. In fishes and gastropods, behavioural changes resulting from elevated CO_2 exposure have been linked to impaired function of $GABA_A$ receptor neurotransmitters (e.g., Nilsson *et al*., 2012; Hamilton *et al*., 2014; Watson *et al*., 2014). In squids, $GABA_A$ receptors are in part responsible for function of the body pattern displays as they are closely tied to the contracting of chromatophores (Kinoshita *et al*., 2013). As the effects of elevated CO_2 on squid behaviours include altered body pattern displays (Spady *et al*., 2014, 2018, **Chapter 4**), it would be worth investigating if elevated CO_2 inhibits $GABA_A$ receptor function in squids as well.

Despite the importance of $GABA_A$ receptors in the learning processes of squids (Conti *et al*., 2013), and the link between CO_2-induced $GABA_A$ impairment and inhibited learning in fish (Chivers *et al*., 2014), I found no effect of elevated CO_2 on the learning or capacity for

conditional discrimination in *S. lessoniana* (**Chapter 5**). This could suggest that changes to squid behaviours from elevated CO_2 in **Chapter 4** are due to a different mechanism. The absence of effects of elevated CO_2 on learning is an encouraging result, as the advanced learning and memory skills of cephalopods are crucial to their ecological success, being important for predation, predator recognition, navigation, social interactions, and much more (Hanlon & Messenger, 2018). However, it is not clear why some ecologically important behaviours are altered by elevated CO_2 (**Chapter 4**), while other equally important behaviours are unaffected (**Chapter 5**).

6.2 – Future directions

This thesis reveals much about the effects of elevated CO_2 on the physiology and behaviours of squids. However, much more needs to be learned in order to predict how squids will be affected by potential future CO_2 levels in the oceans. Firstly, while short-term studies provide some useful understanding to individual stress tolerance, they do not consider the effects of elevated CO_2 over several generations. Squids have short lifespans, rapid growth rates, large populations, and a high intrinsic rate of population increase, which favours adaptations to changing environmental conditions (Boyle & Rodhouse, 2005). However, whether they can adapt to the rapid pace of increasing pCO_2 remains unknown. Cephalopods are generally challenging to rear in captivity. However, advances in the culturing of cephalopods (e.g., Walsh *et al*., 2002), brings answering this question within reach, and doing so is essential in determining what lies ahead for these taxa.

Secondly, elevated CO_2 alters squid behaviours in some ecologically important traits, yet others are not affected. With the use of certain drugs, the relationships between these

altered behaviours and various neurological mechanisms can be determined, for example by using GABA antagonist drugs to explore the effects on $GABA_A$ receptors (Nilsson *et al*., 2012). Furthermore, the molecular response of squids could be determined during short-term, developmental, and transgenerational CO_2 exposure. By examining gene expression during these stages and corresponding altered behaviours, we can gain a better understanding of how and why elevated CO_2 is affecting specific behaviours (e.g., Schunter *et al*., 2018). Determining exactly what is responsible for these changes in behaviour as a result of elevated CO_2 allows for the effects of elevated CO_2 on marine animal behaviours to be more accurately predicted and understood.

Finally, while elevated CO_2 by itself is a risk to marine ecosystems, the ocean is also warming rapidly, putting marine animals at further risk. It will be important for future studies to test the effects of elevated CO_2 in conjunction with elevated temperatures. It has been shown that the adverse effects of projected near-future elevated CO_2 and warming on squid development can be exacerbated when these stressors are tested in combination compared to individually (Rosa *et al*., 2014). However, in other instances, warming has been found to offset some of the negative effects of elevated CO_2 (Lefevre, 2016). Establishing if elevated CO_2 and warming have additive, synergistic, or antagonistic effects on squid physiology, development, and behaviour will be critical for making reliable predictions about the impacts of climate change on cephalopods. Furthermore, diel variations in CO_2 concentrations have been shown to reduce the severity of behavioural abnormalities in coral reef fish at elevated CO_2 (Jarrold *et al*., 2017). Future studies on the effects of elevated CO_2 on both the behaviours and physiology of squids should take into account these fluctuating conditions within the animal's habitat. If animals are found in areas with very high diel variations in CO_2

concentrations, they may be well suited to adapt to the changes projected for the end of the century. Examining the responses of these animals in increasingly realistic and ecologically relevant scenarios will provide better predictions of their future in our changing oceans.

6.3 – Concluding remarks

This thesis provides novel insights into the effects of elevated CO_2 on squid physiology and behaviour. To date, these are the first studies to investigate the effects of elevated CO_2 on aerobic recovery, reproduction, growth and development after parental exposure, predatory behaviours, and learning in any cephalopod species. What has been revealed in these data chapters, in both physiological and behavioural responses, is comparable effects of elevated CO_2 on two separate taxonomic orders of cephalopod. If a wider range of cephalopods are adversely affected by elevated CO_2 in ways similar to those observed here, the consequences could be far reaching. Cephalopods occupy a variety of habitats and communities in which they serve an important role as both predator and prey. Changes to their reproduction, development, and predatory behaviours, have the potential to impact marine ecosystems globally.

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Supplementary Material

Table S1. Results of generalised linear mixed models (*I. pygmaeus*) and generalised linear models (*S. lessoniana*) for all fixed factors. An asterisk (*) indicates $p<0.05$.

Species	Response variable	Fixed factor	χ^2	p-value
I. pygmaeus	$\dot{M}O_{2Max}$	Treatment	0.112	0.738
		Jets	0.028	0.863
		Inks	0.244	0.621
	Routine $\dot{M}O_2$	Treatment	0.188	0.170
	Aerobic scope	Treatment	0.929	0.335
		Jets	0.255	0.613
		Inks	1.781	0.182
	Recovery time	Treatment	0.006	0.936
		Jets	0.260	0.610
		Inks	0.540	0.462
S. lessoniana	$\dot{M}O_{2Max}$	Treatment	0.012	0.915
		Jets	0.414	0.520
	Routine $\dot{M}O_2$	Treatment	0.084	0.772
	Aerobic scope	Treatment	0.607	0.436
		Jets	0.338	0.561
	Recovery time	Treatment	0.083	0.774
		Jets	0.096	0.757

Table S2. Results of generalised linear models for all fixed factors. An asterisk (*) indicates $p<0.05$.

Response variable	Fixed factor	χ^2	p-value
Mean clutch size	CO_2 treatment	8.096	0.004*
	Paternal mass	2.231	0.135
	Maternal mass	1.914	0.166
	Clutch number	1.097	0.295
	Substrate type	0.006	0.937
Distance between eggs	CO_2 treatment	30.925	<0.001*
	Paternal mass	13.633	<0.001*
	Maternal mass	16.397	<0.001*
	Clutch number	0.010	0.922
	Substrate type	0.172	0.678
Percent fertile	CO_2 treatment	1.975	0.160
	Paternal mass	1.776	0.183
	Maternal mass	0.701	0.403
	Clutch number	3.189	0.074
	Substrate type	0.233	0.630
Hatching success	CO_2 treatment	0.004	0.947
	Paternal mass	0.544	0.461
	Maternal mass	0.157	0.692
	Clutch number	0.002	0.961
	Substrate type	0.634	0.426
Time to hatch	CO_2 treatment	0.091	0.763

	Paternal mass	0.123	0.720
	Maternal mass	0.076	0.782
	Clutch number	0.011	0.917
	Substrate type	0.267	0.605
Hatching period	CO_2 treatment	0.196	0.658
	Paternal mass	2.531	0.112
	Maternal mass	0.409	0.523
	Clutch number	1.039	0.308
	Substrate type	0.119	0.730
Embryo size: stage 1	CO_2 treatment	79.786	<0.001*
	Paternal mass	1.645	0.120
	Maternal mass	1.067	0.302
	Clutch number	0.179	0.672
	Substrate type	33.556	<0.001*
Embryo size: stage 23	CO_2 treatment	24.940	<0.001*
	Paternal mass	0.512	0.474
	Maternal mass	0.732	0.392
	Clutch number	0.025	0.874
	Substrate type	5.686	0.017*
Embryo size: stage 30	CO_2 treatment	39.040	<0.001*
	Paternal mass	1.272	0.259
	Maternal mass	42.158	<0.001*
	Clutch number	31.5577	<0.001*
	Substrate type	46.024	<0.001*
Deformed embryos	CO_2 treatment	0.365	0.546
	Paternal mass	0.732	0.392
	Maternal mass	0.190	0.663
	Clutch number	6.931	0.008*

Table S3 - The number of animals used at each CO_2 treatment level for behavioural analysis of each tested trait. Sample sizes for predatory behaviours are less than the total of animals tested because not all animals attacked the prey. Due to occasional logistical issues while recording (e.g. camera angle) not all predatory traits could not be quantified in every trial, resulting in smaller sample sizes for some traits.

Species	***I. pygmaeus***			***S. lessoniana***	
CO_2 treatment	**Control**	**Moderate-CO_2**	**High-CO_2**	**Control**	**High-CO_2**
Total sample size	47	49	47	19	21
Total attacks	39	32	30	18	17
Time to attack	39	32	30	18	17
Pose held	38	31	30	18	17
Strike distance	38	30	29	18	17
Body pattern	37	30	30	18	17
Attack direction	36	30	30	18	17
Activity levels	n/a	n/a	n/a	18	20

Table S4. Results of generalised linear mixed models (*I.* pygmaeus) and generalised linear models (*S. lessoniana*) for all fixed factors. An asterisk (*) indicates $p<0.05$.

Species	Response variable	Fixed factor	χ^2	p-value
I. pygmaeus	**Proportion of attacks**	CO_2 treatment	4.441	0.035*
		Sex	0.107	0.743
		Size ratio	1.511	0.219
	Latency to attack	CO_2 treatment	7.639	0.006*
		Sex	1.184	0.277
		Size ratio	0.029	0.865
	Time holding attack pose	CO_2 treatment	2.899	0.089
		Sex	0.112	0.738
		Size ratio	0.114	0.735
	Striking distance	CO_2 treatment	6.529	0.011*
		Sex	0.724	0.395
		Size ratio	2.789	0.095
	Body pattern choice	CO_2 treatment	5.571	0.018*
		Sex	1.829	0.176
		Size ratio	0.730	0.393
	Attack direction	CO_2 treatment	4.260	0.039*
		Sex	0.310	0.578
		Size ratio	0.379	0.538
	Capture success	CO_2 treatment	0.039	0.843
		Sex	0.767	0.381
		Size ratio	0.003	0.954
S. lessoniana	**Proportion of attacks**	CO_2 treatment	1.809	0.179
		Prey CO_2	2.931	0.087
		Sex	0.220	0.639
		Size ratio	0.076	0.782
	Latency to attack	CO_2 treatment	5.167	0.023*
		Prey CO_2	0.031	0.861
		Sex	0.820	0.365
		Size ratio	0.201	0.654
	Time holding attack pose	CO_2 treatment	0.952	0.329
		Prey CO_2	0.681	0.409
		Sex	2.022	0.155
		Size ratio	0.613	0.434
	Striking distance	CO_2 treatment	1.319	0.251
		Prey CO_2	1.214	0.271
		Sex	1.346	0.246
		Size ratio	2.917	0.088
	Body pattern choice	CO_2 treatment	12.123	<0.001*
		Prey CO_2	0.762	0.383
		Sex	0.102	0.750
		Size ratio	0.091	0.762
	Attack direction	CO_2 treatment	2.059	0.357
		Prey CO_2	0.586	0.746
		Sex	0.379	0.827
		Size ratio	4.561	0.102
	Capture success	CO_2 treatment	1.499	0.221
		Prey CO_2	3.285	0.070
		Sex	4.019	0.045*
		Size ratio	3.745	0.053

	Distance Moved	CO_2 treatment	20.647	<0.001*
		Sex	0.005	0.952
		Squid mass	22.697	<0.001*
	Speed	CO_2 treatment	18.536	<0.001*
		Sex	0.016	0.901
		Squid mass	21.017	<0.001*
	Time Active	CO_2 treatment	14.414	<0.001*
		Sex	0.175	0.675
		Squid mass	11.645	<0.001*